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MICROBIAL SULFOLANE DEGRADATION BY ENVIRONMENTAL ORGANISMS ISOLATED FROM CONTAMINATED SOUR GAS PLANT SEDIMENTS

by

ELIZABETH ANNE GREENE ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Microbiology and Biotechnology
Department of Biological Sciences

Edmonton, Alberta
Fall, 1999

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled MICROBIAL SULFOLANE DEGRADATION BY ENVIRONMENTAL ORGANISMS ISOLATED FROM CONTAMINATED SOUR GAS PLANT SEDIMENTS submitted by ELIZABETH ANNE GREENE in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in MICROBIOLOGY AND BIOTECHNOLOGY.

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ABSTRACT

Sulfolane (tetrahydrothiophene-1,1-dioxide) is used in the Sulfinol® process for natural gas sweetening, which has been implemented worldwide. Sulfolane has entered groundwaters and aquifers through spills, landfills and unlined surface storage ponds. Due to its high water solubility sulfolane poses a risk for off-site contamination.

Three sites were screened in laboratory microcosms to determine the sulfolane degradation potential in contaminated aquifer sediments. All samples degraded sulfolane aerobically. Anaerobic sulfolane biodegradation occurred in some NO_3^-- and Mn(IV)-reducing microcosms.

Aerobic sulfolane biodegradation was further evaluated. Microbial communities from wetlands samples degraded sulfolane without added nutrients. Pristine aquifer sediment samples developed sulfolane-degrading communities if mixing was provided. Contaminated sediment populations that were not supplemented with nutrients eventually degraded sulfolane. Microbial communities from a contaminated aquifer were stimulated by P addition, while subsurface sediments from a different location required N supplementation. Overall, supplementation with the most limiting nutrient improved biodegradation, which was further improved by addition of the nutrient that demonstrated less importance.

A differential medium was developed to detect sulfolane degraders. Sulfolane degraders were detected by the production of acid during aerobic mineralization of the compound. Organisms that were unable to degrade sulfolane produced ammonium from tryptone. This medium was 93% accurate for distinguishing between degraders and nondegraders.

Mixed bacterial cultures converted sulfolane-C to 77 % CO_2, 10 % biomass and 15 % soluble organic compounds, and 82 % of sulfolane-S to $SO_4^{=}$. Pure cultures

produced an average of 38 % CO_2, 23 % biomass, 40 % soluble organic compounds and 59 % $SO_4^=$ from sulfolane C and S, suggesting that some metabolites remained. These were not identified.

A sulfolane-degrading isolate was identified, using a combination of 16S rRNA sequencing, physiological traits and colony and cell morphology. This organism most closely resembled *Variovorax paradoxus*; it may belong to the species *paradoxus*, or it may represent an unidentified species in the genus *Variovorax*.

TO

MY MOTHER

MARGARET PATRICIA MOONEY GREENE

AND

MY HUSBAND

MATTHEW STEPHEN HICKS

THANK YOU FOR ALL YOUR SUPPORT

ACKNOWLEDGEMENTS

I would like to acknowledge the financial support for my research by the Natural Science and Engineering Research Council of Canada and the Canadian Association of Petroleum Producers. I would also like to acknowledge my personal funding from the University of Alberta, the Alberta Heritage Fund and the Alberta Research Council.

Most importantly, I would like to thank Dr. P.M. Fedorak for allowing me to join his group. He has taught me a great deal about research and scholarship, and has endlessly provided me with scientific and personal support, excellent proofreading and superhumanly patient explanations of chemistry. Working in his laboratory is like being apprenticed to a kind uncle. I would also like to thank the members of my committee: Dr. M.J. Dudas, Dr. J.M. Foght and Dr. M.A. Pickard, for their assistance, scientific discussions, and for helping me enjoy my research.

Lisa Gieg welcomed me to the laboratory and has been a wonderful friend, as well as a teacher and collaborator on the initial site research. Her cheerful nature makes work pleasant for everyone. Perrin Beatty also has been a great friend, as well as a coconspirator and coworker on a number of projects, and was of infinite assistance with bacterial identification techniques, posters, company, and general sanity maintenance. Lee Beaudette taught me a number of new techniques. Annie Wong and Sara Ebert have been particularly friendly and helpful, and Sara assisted with some research. Kevin Kropp and Dave Bressler kindly assisted in explaining analytical techniques. Others in the laboratory who made working a pleasure were Cheryl Gauthier and Ken Elder. Tom Hantos and Rich Mah assisted with a number of laboratory techniques and helped out with equipment (particularly the computers...thanks, Rich). Debbi Coy participated in the first two site-related projects and was responsible for some setup and sampling.

A number of people have helped me with my work from a personal point of view. My mother has always encouraged me to pursue my goals, and has always been interested in everything I do. Matt Hicks has been a pillar of support, and always will be. Annette Rohr, the staunchest of old friends and supporters, has been with me since our early years, and has provided excellent field advice. My father provided me with a great deal of assistance in the pursuit of my education over the years. Ned Beatty has been a good friend, and has freely lent me his wife's time and friendship. Helen Ible welcomed Matt and me to Edmonton with open arms at 1 am in a blizzard. My other good friends, you know who you are...Diana Cooper, Jamie Whittla, Kristin Scott, Linda Sandercock, Fred and Maggie Cooper...thanks for your love, friendship and all that good cheer!

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LIST OF ABBREVIATIONS AND ALTERNATIVE TERMS

16s rRNA	small subunit of bacterial ribosomal RNA
^{1}H-NMR	proton nuclear magnetic resonance
2,4-D	2,4-dichlorophenoxyacetic acid
AED	atomic emission detector
amp^R	ampicillin resistant
ATCC	American Type Culture Collection
BLAST	basic local alignment search tool
BSA	N,O-bis(trimethylsilyl)acetamide
BTEX	benzene, toluene, ethylbenzenes, xylenes
CAPP	Canadian Association of Petroleum Producers
cm^R	chloramphenicol resistant
DIPA	diisopropanolamine
DNA	deoxyribonucleic acid
EMB	eosin methylene blue
FID	flame ionization detector
FPD	flame photometric detector
GC	gas chromatography or gas chromatograph
GC-MS	gas chromatography-mass spectrometry
GK	minimal medium containing glucose, sulfolane and kanamycin
HP	Hewlett-Packard
HPLC	high performance liquid chromatography
kan^R	kanamycin resistant
LB	Luria-Bertani medium
LD$_{50}$	dose at which 50% of test organisms are killed within 24 h
μS	microSieverts
NCIMB	National Collections of Industrial and Marine Bacteria, Limited
PAH	polyaromatic hydrocarbon
PAT	pH agar for tetrahydrothiophene-1,1-dioxide
PCA	plate count agar
PCB	polychlorinated biphenyl
PCP	pentachlorophenol
PCR	polymerase chain reaction

RDP	Ribosomal Database Project
RNA	ribonucleic acid
TCD	thermal conductivity detector
TEA	terminal electron acceptor
tet^R	tetracycline resistant

1 INTRODUCTION

1.1 Sour natural gas

Physical and chemical processes such as thermally induced decomposition of organic compounds containing S or thermally catalyzed $SO_4^=$ reduction can contribute to H_2S production in oil and natural gas reservoirs (McInerney et al., 1992); so can microbial reduction of oxidized S compounds. Oil and gas reservoirs in Alberta contain H_2S; this compound is present throughout both the shallow deposits in the southeastern part of the province and in the deeper deposits located in the southwestern mountainous part of the province (Tissot and Welte, 1984).

Oil formation begins with the process of diagenesis, when organic matter is condensed, to eventually form kerogen. The next stage in a developing oil reservoir is catagenesis. At this time the oil reservoir temperature increases, reaching 50 to 150°C, pressure increases, and thermal degradation of kerogen results in the production of liquid petroleum (Tissot and Welte, 1984). During metagenesis, temperature and pressure increase further. No further petroleum production occurs; thermal compression and degradation occur. In this stage, methane is produced (Tissot and Welte, 1984). Sulfide can be produced abiotically late in catagenesis and during metagenesis (Tissot and Welte, 1984). It is the product of thermal cracking of heavy oil and gas components, and is formed mainly in gases that were originally enriched in S compounds. Sulfide is also produced from the reaction of methane with oxidized S compounds at high temperatures, and during volcanic activity (Selley, 1998; Tissot and Welte, 1984). In western Canada, oil and gas reservoirs are enriched in sulfide. The relative contribution of chemical and microbiological processes to this enrichment is not known (Tissot and Welte, 1984).

Many natural gas reservoirs are unlikely to become sour until they have come into contact with agents that might induce souring (Cord-Ruwisch et al., 1987). A substantial amount of H_2S production in the subsurface is the result of microbial activity; anaerobic respiration with $SO_4^=$ as the terminal electron acceptor results H_2S production (Iverson and Olson, 1984). Some bacterial species are able to reduce thiosulfate or sulfite (McInerney and Sublette, 1997); these organisms can also contribute to the souring of oil and natural gas reservoirs and may even be predominant in some locations. Initially sweet reservoirs can gradually sour over a period of years as they are exploited; this souring may indicate the development of $SO_4^=$-reducing bacteria in the well (Cord-Ruwisch et al., 1987). Contamination of wells with these organisms likely occurs during drilling and oil recovery. Further contamination is possible when surface or groundwaters are injected into the well to improve production.

The presence of H_2S in oil and gas, regardless of its source, is responsible for corrosion of iron-containing materials such as oil recovery equipment, pipelines, and storage tanks in the oil and gas industry, and the presence of H_2S can also lead to the corrosion of concrete. Hydrogen sulfide is highly toxic, and is responsible for a number of deaths in Canada and the United States each year. Combustion of S-containing compounds can result in air pollution; stringent regulations concerning the S content of fuel exist in many countries (Chong, 1994; Selley, 1998). Removal of H_2S, CO_2 and other gas souring compounds, such as mercaptans, COS and CS_2, from oil and natural gas is therefore critical for the industry.

1.2 Sulfinol® process for sour gas treatment

Sweetening of natural gas is required before it can be sold. Depending on the concentration of H_2S, COS, CS_2, CO_2, mercaptans and other sour components present, different sweetening processes are employed for gas treatment. At low concentrations of gas souring compounds, chemical processes are advantageous. As the partial pressure of the gas souring compounds increases, chemical agents become saturated, at which point their capacity becomes limited. At these concentrations, physical solvents become more useful because they are not dependent on a 1:1 relationship with the souring compounds, whereas chemical reagents are required to be present in stoichiometric amounts with the souring compounds (Astarita et al., 1983; Goar, 1971; 1976; Kohl and Riesenfeld, 1979).

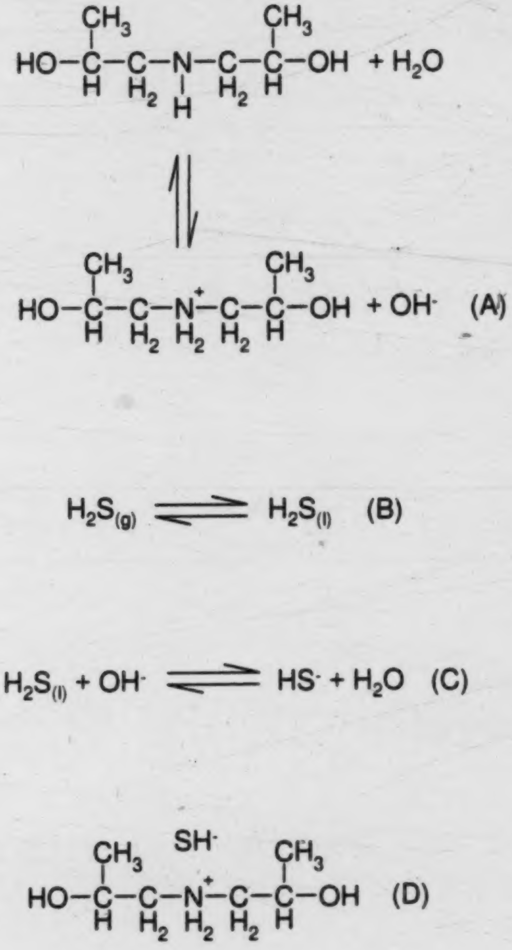
Chemical processes for gas sweetening involve a chemical interaction between the sour components in oil or natural gas and the sweetening compound. Several different amine compounds are commonly used in these processes, depending on the type and concentration of the gas souring compounds present; most are alkanolamines (Goar, 1971). Physical gas sweetening processes involve dissolving the sour components in a physical solvent to separate them from the gas. The advantage of using a chemical sweetening process is that it is highly specific for sour components, however the interaction between sour components and the chemical solvent becomes rate limiting as sour component concentrations increase. In contrast, physical solvents remove sour components by physical adsorption; therefore they are not limited by chemical interactions between these components and the sweetening chemicals. All the physical solvents used in gas sweetening processes are organic solvents (Goar, 1976).

A problem with physical solvents is that some heavy (>C_5) hydrocarbons, particularly aromatic and olefin compounds, tend to enter the solvent phase. The absorbed hydrocarbons interfere with S recovery later in the treatment process, which means that an activated C filtration step must be added to the solvent treatment process to remove them (Goar, 1971; 1976; Kohl and Riesenfeld, 1979). In spite of this added step, physical processes can be useful when conditions warrant (Goar, 1976).

The Sulfinol® gas sweetening method involves a combination of physical and chemical processes. This particular process is most effective when the H_2S:CO_2 ratio is greater than 1:1 and sour gas has a relatively high acid gas partial pressure (Goar, 1971; 1976; Maddox, 1977). The Sulfinol® gas sweetening process utilizes diisopropanolamine (DIPA) as the chemical solvent. DIPA interacts with sour gas components via its central N; it dissociates in water and acts as a counterion in solution (Figure 1.1).

Sulfolane acts as the physical solvent in the Sulfinol® process. The presence of sulfolane in the gas sweetening solution allows increased CO_2 absorption over an amine and water mixture. Sulfolane is also stable at high temperatures, thus the Sulfinol® solvent can be regenerated by heating. Dissolved hydrocarbons and sour gas compounds are stripped from the solvent during the heating process, allowing the solvent to be recycled. Regeneration of the solvent is important because sulfolane is relatively expensive (Andersen et al., 1977).

The Sulfinol® process was introduced in the early 1960's, and since that time it has been implemented in a large number of gas sweetening plants worldwide; Kohl and Riesenfeld (1979) reported over 100 plants in operation at that time. Fitzgerald and Richardson (1966) describe the use of the Sulfinol® process at the Harmattan plant (near Olds, AB); this was the first sweetening plant in western Canada designed to remove H_2S from sour gas. Initially the Sulfinol-D process was used (Taylor et al., 1991). This solvent system contained sulfolane, DIPA and water in approximately 35:45:20 ratios by percent weight (Goar, 1976); the exact ratios vary between plants. In the 1980's, the Sulfinol-M solvent was developed. This system uses methyldiethanolamine (MDEA) as the chemical solvent rather than DIPA, and has a much higher specificity for H_2S over CO_2 (Taylor et al., 1991). This is important as regulations concerning CO_2 content in gas often differ from those concerning H_2S; some CO_2 is often permitted to remain in the final product. Regardless of the amine component used, sulfolane is always present in Sulfinol® process solvents. The initial use of DIPA with sulfolane has



$$HO-CH(CH_3)-CH_2-NH-CH_2-CH(CH_3)-OH + H_2O \rightleftharpoons HO-CH(CH_3)-CH_2-N^{+}H_2-CH_2-CH(CH_3)-OH + OH^- \quad (A)$$

$$H_2S_{(g)} \rightleftharpoons H_2S_{(l)} \quad (B)$$

$$H_2S_{(l)} + OH^- \rightleftharpoons HS^- + H_2O \quad (C)$$

$$HO-CH(CH_3)-CH_2-N^{+}H_2-CH_2-CH(CH_3)-OH \;\; SH^- \quad (D)$$

Figure 1.1. Interaction between diisopropanolamine (DIPA) and H_2S. A. DIPA is a weak base, and will dissociate in water. The pH of a 5 % solution of DIPA in water, is approximately 11.5. B. H_2S gas dissolves in aqueous solution. C. H_2S loses proton to OH^- from reaction (A). D. Hydrosulfide and protonated DIPA are counterions in solution. Information was adapted from a discussion of DIPA and H_2S by Astarita et al. (1983).

resulted in co-contamination of these two compounds at most Sulfinol® plants where soil and groundwater contamination has occurred in the past.

Sulfolane is only lost from Sulfinol® process reactors through vaporization, however a small amount of DIPA is lost through conversion to other products, such as DIPA-oxazolidone (Goar, 1976; Maddox, 1977; Taylor et al., 1991). Both sulfolane and DIPA are regenerated and recirculated through the treatment process, and therefore must be filtered (Maddox, 1977). Sulfolane, DIPA and other compounds such as dissolved hydrocarbons and DIPA-oxazolidone have leached into groundwaters and sediments on gas sweetening plant sites due to solvent spills, unlined process water ponds and landfills containing filters, spent solvent and other wastes.

1.3 Properties of sulfolane

Sulfolane (Figure 1.2) is known by many chemical names, including: bondelane A, bondolane A, cyclotetramethylenesulfone, dihydrobutadiene sulfone, dioxo-2,3,4,5-tetrahydrothiophene, 1,1-dioxothiolane, 1-1-sulfalone, sulfolan, sulfoxaline, sulpholane, tetrahydrothiophene-1,1-dioxide, 2,3,4,5-tetramethylene sulfone, and thiocyclopentane-1,1-dioxide (P.M. Fedorak, personal communication). The melting point is 27.4 to 27.8°C, the boiling point is 280 to 285°C, and the liquid density of sulfolane is 1.2606 (Merck Index, 1968).

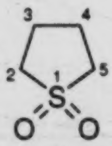


Figure 1.2. Chemical structure of sulfolane.

Sulfolane is a relatively inert chemical; it does not react with acids or bases (Brown et al., 1966). It is also thermally stable (Chou and Swatloski, 1983). Sulfolane is an excellent solvent for most types of organic chemical compounds (Chou and Swatloski, 1983) and is therefore frequently used as a solvent for industrial purposes. Its thermal stability allows it to be re-used in industrial processes; for instance the Sulfinol® solvent is regenerated through heating (Andersen et al., 1977). As well as being used for gas sweetening processes, sulfolane is used to extract aromatics from gas, separate low-boiling point alcohols, fractionate wood tar, tallow oil and fatty acids, concentrate

SO_2, and to dehydrate solutions (Chou and Swatloski, 1983). The chemical stability of sulfolane allows it to be used as a solvent in analytical processes such as fast atom bombardment-mass spectrometry (Dass, 1990) and nuclear magnetic resonance (Kopple et al., 1992).

Sulfolane is miscible in water, acetone, glycerol and toluene (Brown et al., 1966), and partially miscible with octanes, olefins and naphthenes (Merck Index, 1968). It is therefore useful as a selective solvent for liquid-vapor extractions (Merck Index, 1968).

Known examples of sulfolane in the environment tend to involve sites of industrial contamination; for the most part sulfolane is recognized as a manmade chemical, however this compound has been found to occur naturally in one source. A composite of a *Batzella* sp. sponge and a *Lissoclinum* sp. tunicate found on Point Impossible, Victoria, Australia, has been demonstrated to produce sulfolane. The majority of the chemistry associated with both these species apparently involves S-containing compounds (Barrow and Capon, 1992). These authors were unable to find other examples of sulfolane as a naturally occurring compound.

1.4 Toxicity of sulfolane

Industrial processes involving sulfolane have existed for some time; therefore studies on its toxicity have been done. Initially, sulfolane toxicity due to ingestion, injection or inhalation was examined. Similar LD_{50} values were found by a number of authors for orally administered or subcutaneously injected sulfolane, in different animals. These are summarized in Table 1.1.

Although the LD_{50} of sulfolane is relatively high in the laboratory animals tested, it has marked central nervous system effects at lower doses. At lethal doses, animals convulsed and demonstrated difficulty in breathing before death (Alexander et al., 1959, Andersen et al, 1976; Brown et al., 1966). Andersen et al. (1976) determined that sulfolane had an effect on the central nervous system, which was demonstrated by the convulsions that the animals experienced. Later studies have also suggested central nervous system effects due to sulfolane (Ruppert and Dyer, 1985).

Sulfolane did not appear to have toxic systemic effects when applied to the skin, nor did it cause skin irritation (Brown et al., 1966). These authors were also unable to detect any development of sensitivity to sulfolane in guinea pigs after repeated exposures of shaved skin patches to the compound. Ursin et al. (1995) found that living human skin showed little permeability to sulfolane (0.2 $g/m^2/h$), which likely explains the

Table 1.1. LD_{50} values for sulfolane administered to animals by ingestion, subcutaneous injection or dermal exposure.

type of animal tested	method of sulfolane administration	LD_{50} g/kg	source of information
mouse	oral	1.9 to 2.5	Brown et al., 1966
	injected	1.1	Alexander et al., 1959
	injected	0.6 to 1.4	Andersen et al., 1976
rat	oral	2.1	Brown et al., 1966
	oral	1.8	Andersen et al., 1976
	injected	1.1 to 1.6	Andersen et al., 1976
	dermal	>3.8	Brown et al., 1966
guinea pig	oral	1.8	Andersen et al., 1976
	injected	1.3	Andersen et al., 1976
rabbit	injected	0.6 to 3.5	Andersen et al., 1976

lack of systemic toxicity of the compound via this route. Direct eye exposure to sulfolane caused temporary irritation in rabbits, but had no lasting effects (Brown et al., 1966).

The inhalation toxicity of sulfolane was also examined (Andersen et al., 1977). These authors found that it was not possible to determine an LD_{50} for inhaled sulfolane. When it was vaporized into air, sulfolane concentrations that condensed onto the animals and cages were reached before the LD_{50} could be measured. At this point it was impossible to distinguish between inhaled and ingested sulfolane. However, sublethal exposure to vaporized sulfolane had marked effects on laboratory animals. Some of these results are summarized in Table 1.2.

Sulfolane appears to affect laboratory animals at both a physiological and a behavioral level. Ruppert and Dyer (1985) noted that subcutaneous injection of sulfolane caused animals to experience hypothermia, and that at colder ambient temperatures the hypothermia and resultant decrease in motion of the animal appeared to be protective against the toxic effects of sulfolane, to some extent. Animals generally showed a decrease in activity when injected with sulfolane.

Sulfolane causes central nervous system stimulation, resulting in seizures at high doses (Alexander et al., 1959; Andersen et al., 1977; Brown et al., 1966). It also causes severe lung irritation, and in some cases causes animals to vomit when administered orally or inhaled (Andersen et al., 1977; Brown et al., 1966). In general, the effects of sulfolane appeared to be greatest in monkeys, and to decrease respectively in dogs, rabbits, guinea pigs, rats and mice (Andersen et al., 1977). This evidence suggests that sulfolane would affect humans to a greater extent than some of the smaller test animals such as rodents.

When sulfolane was introduced to the bloodstream of rats or rabbits, some was enzymatically hydroxylated to 3-hydroxysulfolane, while the remainder was unconverted (Roberts and Warwick, 1961). Andersen et al. (1976) discovered that if 100 mg/kg of ^{35}S-sulfolane was administered to rats, 85% would be excreted in the urine as ^{35}S-3-hydroxysulfolane within 24 h; the remainder was excreted as sulfolane. With increasing quantities of ^{35}S-sulfolane administered, the amount of excreted ^{35}S-3-hydroxysulfolane remained constant, while the proportion of ^{35}S-sulfolane excreted increased. Sulfolane appeared to have a half-life in the animal's bloodstream of 3 to 5 h (Andersen et al., 1976). The rapid excretion of this compound may explain why large doses are required before toxic effects occur.

Table 1.2. The effects of sulfolane inhalation on laboratory animals.[a]

animal	sulfolane (mg/m^3)	exposure time	effect on animals
rat	3600	17.5 h	convulsions, decrease in white blood cells, pulmonary hemorrhage
	495	8 h/d, 5 d/wk	chronic lung and liver inflammation, decrease in white blood cells
	2.8	continuous, 90 d	no toxic signs
guinea pig	495	8 h/d, 5 d/wk	chronic lung inflammation
	200	continuous, 90 d	chronic lung inflammation, fatty liver metamorphosis
	2.8	continuous, 90 d	no toxic signs
dog (beagle)	200	continuous, 90 d	intermittent convulsions, extreme aggression, vomiting, chronic lung hemorrhage
	2.8	continuous, 90 d	no toxic signs
squirrel monkey	4850	18.5 h	vomiting, convulsions, decrease in white blood cells, pulmonary hemorrhage
	495	8 h/d, 5 d/wk	death, fatty metamorphosis of liver, lung inflammation, decrease in white blood cells
	2.8	continuous, 90 d	no toxic signs

[a] From Andersen et al. (1977).

Animal studies demonstrated that sulfolane was hydroxylated at the 3-position. Roberts and Warwick (1961) proposed that a more chemically favorable reaction would be hydroxylation at the 2-position; when sulfolane is subject to a nucleophilic attack it is hydroxylated at the 2-position (Faith, 1953). The authors proposed that the 3-hydroxylation of sulfolane, rather than 2-hydroxylation, would be the product of an enzymatic reaction because the latter product would be unlikely due to steric hindrance from the sulfone group.

1.5 Migration of contaminants in groundwater

The migration of contaminants from a given pollutant site into an aquifer via groundwater is governed, for the most part, by two factors: the water solubility of the compound and its attenuation in soil or aquifer sediments. Compounds that are highly water-soluble can travel much more readily in groundwater; particularly compounds that are solid at the ambient aquifer temperature, since they will become dissolved (Morgan and Watkinson, 1989). Leachate (the portion of the contaminant plume soluble in groundwater) tends to travel in the direction of the groundwater flow, with little horizontal spread perpendicular to the groundwater flow; leachate plumes are rarely much wider than the contaminant source itself (Christensen et al., 1994). The density of contaminants dissolved in groundwater will also affect their migration through an aquifer; increasing density will increase the downward flow of the contaminant plume (Christensen et al., 1994). Insoluble compounds can move quickly through an aquifer on the surface of the water table, however they will not have the same mobility throughout the entire aquifer as more water-soluble compounds (Morgan and Watkinson, 1989).

Attenuation of contaminant compounds can occur by physical interactions between soil and the compounds, or by chemical or biological alteration of the contaminants. Increases in interactions between soil and contaminant compounds will decrease the rate of migration through an aquifer. Hydrophobic materials, such as polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), and benzene, toluene, ethylbenzene and xylene (BTEX compounds) can interact with the soil humic material (Barker et al., 1987; Madsen, 1991; Morgan and Watkinson, 1989; Sims et al., 1990). Charged molecules can interact with charged particles present in soil organic matter, but will also interact with charged compounds that make up part of the clay mineral content of soil (Luther et al., 1998; Sims et al., 1990). Larger molecules will interact more strongly with soil via Van der Waals forces than smaller molecules, and molecules containing electronegative atoms can interact with soil components by hydrogen bonding (Sims et al., 1990). Biodegradation of organic compounds in an aquifer will depend on the microbial population and availability of nutrients such as N, P and terminal electron acceptors. The effects of nutrient availability are discussed in Section 1.9. The availability of terminal electron acceptors in an aquifer will affect its redox state as well as the biological activity against contaminant compounds. The effects of this availability are discussed in Section 1.6.

1.6 Organic compound oxidation under different redox conditions

As biodegradable organic compounds migrate through an aquifer, some of the compounds in this plume may be utilized. Oxidation of organic compounds yields different amounts of energy, depending on the terminal electron acceptor used. Oxygen as a terminal electron acceptor yields the most energy, CO_2 the least. The average free energy changes for the oxidation of the model compound CH_2O, utilizing different terminal electron acceptors, are shown in Table 1.3. Although some compounds are more readily degraded or transformed under anaerobic conditions, many are more recalcitrant to attack by microorganisms in the absence of O_2 (Sims et al., 1990). The redox state of a contaminated aquifer is therefore important for determining whether contaminants that have leached into an aquifer can be removed by bioremediation. The terminal electron acceptor of choice for microorganisms will usually be the one which yields the maximum free energy during respiration (Bouwer, 1992). Generally, O_2 is used first at the leading edge of the plume, followed by NO_3^-, then Mn(IV) and Fe(III), followed by $SO_4^=$ and then CO_2 (Gounot, 1994; Nealson and Saffarini, 1994). The resulting redox state of the aquifer is that the most reduced region is next to the contaminant source. As the contaminant plume travels downgradient from the source, the redox potential of the aquifer increases, since fewer of the potential terminal electron acceptors have been exhausted (Bouwer, 1992; Christensen et al., 1994; Sims et al., 1990). A diagram of the redox zones in a model of a leachate plume is shown in Figure 1.3. The different microbial communities observed in different redox zones of a leachate plume tend to support the theory that redox zones are formed by microbiological activity. Where a given terminal electron acceptor appears to be favored, the appropriate microbial population can often be detected (Christensen et al., 1994).

For aerobic biodegradation of contaminant compounds, O_2 can act as both a terminal electron acceptor and as a participant in the chemical reactions involved in compound oxidation (Grbić-Galić, 1990). Oxygen in the groundwater will likely be rapidly utilized by organisms capable of degrading the most easily oxidized contaminant

Table 1.3. The Gibb's free energy change ($\Delta G°$) for degradation of the model compound CH_2O using different terminal electron acceptors (TEAs).[a]

reaction type	TEAs	reaction using model compound CH_2O	$\Delta G°$ (kJ/mol)
aerobic respiration	O_2	$CH_2O + O_2 \rightarrow CO_2 + H_2O$	-502
nitrate reduction	NO_3^-	$5CH_2O + 4NO_3^- + 4H^+ \rightarrow 5CO_2 + 2N_2 + 7H_2O$	-477
manganese reduction	Mn(IV)	$CH_2O + 2MnO_2 + 4H^+ \rightarrow CO_2 + 2Mn^{++} + 3H_2O$	-339
iron reduction	Fe(III)	$CH_2O + 4Fe(OH)_3 + 8H^+ \rightarrow CO_2 + 4Fe^{++} + 11H_2O$	-117
sulfate reduction	$SO_4^{=}$	$2CH_2O + SO_4^{=} + H^+ \rightarrow 2CO_2 + HS^- + 2H_2O$	-105
methanogenesis	CO_2	$2CH_2O \rightarrow CH_4 + CO_2$	-92

[a] The free energy change was calculated for reactions at pH 7. From Bouwer (1992) and Christensen et al. (1994). The $\Delta G°$ values were converted to kJ/mol from kcal/mol using the value 1 cal = 4.186 J (Serway, 1986).

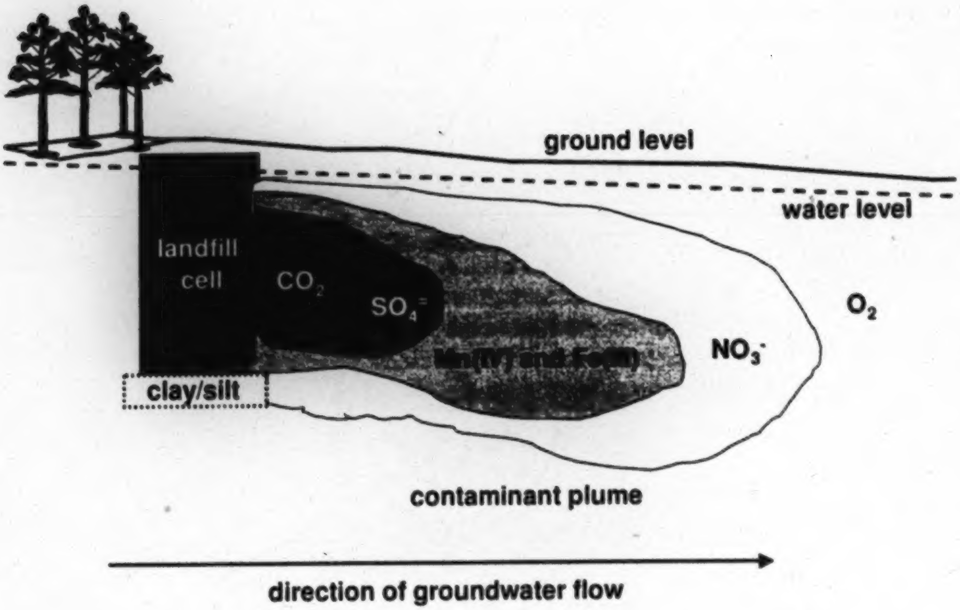


Figure 1.3. Generalized diagram of redox conditions in an aquifer contaminated from a single source. The most energetically favorable terminal electron acceptor for each condition is shown in each region of the plume. Information was taken from Bouwer (1992) and Christensen et al. (1994).

compounds (Christensen et al., 1994). Once O_2 has been depleted, the terminal electron acceptor most likely to yield high levels of energy is NO_3^- (Table 1.3).

The reduction of NO_3^- is thought to occur in a stepwise manner: NO_3^- is reduced to NO_2^-, then to NO, N_2O and finally N_2 (Schulthess et al., 1995). Both NO_2^- and NO can be toxic to bacterial activity; accumulation of these compounds may affect biodegradation detrimentally (Schulthess et al., 1995). The presence of NO_3^- has been demonstrated to inhibit Fe(III) reduction, however it does not appear to be inhibitory to Mn(IV) reduction. Inhibition of Fe(III) reduction by NO_3^- is thought to be due to competition for substrate rather than by any direct activity against Fe(III)-reducing organisms (Lovley, 1991). The higher free energy change yielded by Mn(IV) reduction when compared with Fe(III) reduction (Table 1.3) may explain why Mn(IV) reduction can occur in the presence of NO_3^-.

Both Fe(III) and Mn(IV) reduction can occur simultaneously in aquifer sediments; the major factor governing utilization of either metal as a terminal electron acceptor appears to be its availability (Lovley, 1991; Nealson and Saffarini, 1994). Bacterial cells must come directly into contact with the useable form of Fe(III) or Mn(IV) (Nealson and Saffarini, 1994), and because both these metals can undergo abiotic reduction reactions (Lovley, 1991) or interact with mineral salts or clay minerals and become insoluble, availability of the correct forms of either metal can be limited. In spite of this potential lack of availability, Fe(III) is often a major terminal electron acceptor in contaminated environments (Lovley, 1991). As the presence of NO_3^- can result in the inhibition of Fe(III) reduction coupled to biological oxidation of organic matter, so can the presence of Fe(III) in an environment interfere with $SO_4^=$ reduction and methanogenesis (Lovley, 1991). This interference is probably due to the successful competition for organic growth substrates by Fe(III)-reducing bacteria. Sulfate reduction and methanogenesis yield less energy per substrate molecule than metal reduction (Table 1.3).

The reduction of $SO_4^=$ involves the oxidation of organic compounds or H_2 coupled to the reduction of $SO_4^=$. Other oxidized S compounds such as $SO_3^=$, $S_2O_3^=$ and S°, to H_2S can act as terminal electron acceptors for some $SO_4^=$-reducing organisms (Jørgensen, 1983; Lovley, 1991; McInerney et al., 1992; Postgate, 1979). Sulfate-reducing bacteria are found in groundwaters and freshwater and marine sediments, and are likely responsible for the formation of most H_2S found in these locations (Iverson and Olson, 1984). Methanogenic and $SO_4^=$-reducing bacteria can utilize some of the same substrates; $SO_4^=$-reducing microorganisms may therefore inhibit methanogenesis by competition for available nutrients (Grbić-Galić, 1990). Although methanogenesis yields the least energy per molecule of substrate oxidized of any of the terminal electron acceptors discussed (Table 1.3), it may eventually become the most important activity in a contaminated aquifer, if the other terminal electron acceptors have been exhausted (Grbić-Galić, 1990).

1.7 Migration of sulfolane at contaminated sites

Sulfolane is a polar molecule that exhibits very little chemical reactivity. Luther et al. (1998) determined the sorption coefficients for sulfolane with montmorillonite and kaolinite clays. Montmorillonite has a high cation exchange capacity and large surface area whereas kaolinite has a relatively low surface area and cation exchange capacity. These authors determined that sulfolane interactions with clay minerals were proportional to the cation exchange capacity or surface area of the clay mineral in that montmorillonite was more reactive toward sulfolane than kaolinite. Luther et al. (1998) also measured the interactions between sulfolane and a humus-rich soil and found that these interactions were mainly defined by the montmorillonite clay content of the soil, rather than by any organic components. The distribution coefficient (K_d) of sulfolane in different clays was measured by Luther et al. (1998). The K_d for sulfolane and montmorillonite clay was found to be 0.94 L/kg; for kaolinite the K_d was 0.18 L/kg (Luther et al., 1998). Based on these results, the authors determined that sulfolane mobility in soil is very high. It is therefore probable that at contaminated sites where sulfolane is present in the groundwater, sulfolane could eventually migrate off site and contaminate nearby domestic wells and surface waters.

Sulfolane does not interact significantly with soil organic matter or with the clay mineral portion of soil; therefore its attenuation in contaminated aquifers is likely to be dependent on microbial activity against the compound. Aerobic biodegradation of sulfolane has been demonstrated; this will be reviewed in Section 1.10. There are no reports on anaerobic sulfolane biodegradation in the literature to date. Sulfolane has been observed to migrate further than its co-contaminant DIPA at three sour gas plant sites (J.H. Sevigny, Komex International Ltd, Calgary, AB; personal communication), which suggests that biological attenuation of this compound is not sufficient to prevent its spread in groundwaters and aquifer systems where it has been introduced.

1.8 Bioremediation of contaminated soils and groundwater

Remediation of contaminated aquifers and groundwaters can be carried out by physical, chemical or microbiological means, or by a combination of the above. Chemical and physical remediation are problematic in that they can be expensive when applied to a large site, and can also be disruptive to the site; especially activities like the removal of contaminated soil for incineration or landfilling (Morgan and Watkinson, 1989). *In situ* remediation can be inexpensive relative to physical or chemical cleanup methods. Bioremediation is also advantageous in that contaminant compounds are removed or transformed rather than simply controlled on site or taken to a new site (Ritter and Scarborough, 1995; Rorech and Morello, 1995; Sims et al., 1990; Thomas and Ward, 1989). However, some substances are not easily degraded and may not be removed from a site by biological activity (Madsen, 1991; Mueller et al., 1991; Morgan and Watkinson, 1989), and in other cases the products of biotransformation can be more harmful than the original contaminants (Sims et al., 1990). For instance, the microbially mediated reductive dechlorination of tetrachloroethylene, a commonly used solvent, can result in the production of vinyl chloride, which is much more toxic to humans and to the resident bacterial population than its parent compound (Bouwer, 1992).

The existence of recalcitrant compounds and toxic biodegradation products demonstrate the potential fallibility of bioremediation as a sole means of site cleanup. Reasons for microbial fallibility can include a lack of the appropriate terminal electron acceptors or essential growth factors for substrate removal, low bioavailability of the substrate, toxicity of the substrate, degradation products or co-contaminants, and the inability of microorganisms to adapt degradative enzymes for some man-made chemicals (Alexander, 1965). In spite of the recalcitrance of some compounds, research indicates that microbial activity is able to decrease the concentration of a large number of organic pollutants from soils, aquifers and groundwaters. This activity is site-specific; therefore it is important to assess the biodegradation potential at each site before considering a particular bioremediation strategy (Blackburn and Hafker, 1993).

Bioremediation generally refers to the degradation or removal of contaminant compounds at a given site. This activity can be encouraged by the enrichment of such organisms via nutrient enhancement of the site, or to the addition of bacteria or fungi capable of degrading the compounds of interest to a given site (Lee et al., 1987; Ritter and Scarborough, 1995; Sims et al., 1990). Enrichment of the organisms of interest frequently involves the addition of nutrients such as N, P or O_2. Oxygen increases the energy yield for the degradation of each molecule of organic substrate consumed, thereby increasing the overall microbiological activity against organic compounds. Often aquifers do not contain the amount of nutrients such as N and P that are required for removal of organic contaminants, particularly if the organic C content of a soil is high due to contamination or simply to the physical makeup of the soil aquifer (Morgan and Watkinson, 1989; Ritter and Scarborough, 1995; Sims et al., 1990). Microbial activity in the presence of organic C can quickly exhaust the nutrients required for biodegradation (Sims et al., 1990). In these cases, addition of the appropriate nutrients can increase the rate of removal of contaminant compounds.

Bioremediation can be done *in situ* or *ex situ*. *In situ* remediation techniques include landfarming and related techniques that involve modifying contaminated soils and sediments with nutrients and O_2, phytoremediation, bioventing or biosparging, composting or groundwater treatment with nutrients and O_2 (Alexander, 1999). *Ex situ* techniques rely on transporting contaminated sediments and/or groundwaters to bioreactors or biofilters for treatment (Alexander, 1999). The *ex situ* treatments are more expensive and disruptive than *in situ* bioremediation, and are less practical for sites that are extensively contaminated due to the amount of material requiring treatment.

Bioremediation can often be combined with abiotic techniques to improve contaminant removal. For instance, with *in situ* bioremediation techniques such as bioventing (aerating the unsaturated zone of a conatminated site) or biosparging (aerating the saturated zone), the passage of air through the contaminated soil will remove volatile compounds, which are dispersed into the air. Contaminants can also be removed during *ex situ* treatment; reactive chemicals, UV light or other catalysts can be used to modify compounds or decrease their toxicity before materials are introduced to the bioremediation system.

1.9 The effect of nutrient addition on biodegradation of contaminants in soil and aquifer sediments

The addition of inorganic nutrients, particularly N and P, has frequently been suggested as a means for increasing the biodegradation of contaminant compounds by the resident microbial populations of contaminated sites. Many studies have shown that biodegradation of contaminants is stimulated by the addition of inorganic N and/or P (Braddock et al., 1997; Bragg et al., 1994; Fedorak and Coy, 1996; Lai et al., 1996; Mueller et al., 1991; Piehler and Paerl, 1996).

Braddock et al. (1997) demonstrated that biodegradation of hydrocarbons from JP-5 jet fuel by a microbial community in Arctic soil was mainly limited by the absence of N, however further stimulation of microbial activity occurred after the addition of P, as well as N, to soil microcosms. Laboratory soil microcosms consisted of 400 g contaminated gravel taken from 0.5 m below the soil surface; each microcosm was incubated in a sealed, quart-size Mason jar. *In situ* field mesocosms, each consisting of 20 kg soil in a polyethylene container with drainage holes at the bottom, were also monitored. The authors measured microbial activity in the microcosms and mesocosms over the course of the experiment using radioisotope-labeled naphthalene and hexadecane, and most probable number assays for diesel and gasoline degraders and for total heterotrophs. Total C mineralization was also measured, using Mason jar microcosms, by analyzing CO_2 in the headspace gas using gas chromatography (GC). Laboratory experiments demonstrated that the most significant increase in microbial activity occurred when microcosms were amended with both N and P; the addition of N alone caused a lesser increase in microbial activity, while the addition of P alone had little effect. The mesocosm experiment yielded similar results. Interestingly, it was found that low levels of fertilizer (100 mg/kg N, 45 mg/kg P, per dry weight soil) and medium levels (200 mg/kg N, 90 mg/kg P, per dry weight soil) stimulated microbial populations more than high levels (300 mg/kg N, 135 mg/kg P, per dry weight soil). Braddock et al. (1997) found other instances of inhibition of soil microbial activity at high levels of added N in the literature, therefore their observations were not entirely surprising. Their conclusion was that for the Arctic site under investigation, N was the major limiting factor for hydrocarbon biodegradation, but P, to a lesser extent, also played a role in nutrient limitation. These authors suggested that optimization of bioremediation using fertilizer amendment would be site specific, and should be evaluated on a site by site basis (Braddock et al., 1997).

Bragg et al. (1996) also determined N to be the limiting factor for biodegradation of hydrocarbons. These authors studied the effectiveness of different bioremediation strategies on hydrocarbon degradation using field studies in an area contaminated by the oil spill from the *Exxon Valdez* in Prince William Sound. Three contaminated sites were divided in half; one half was fertilized with N and P in a 10:1 ratio and the other half was left untreated. Sampling wells were placed in each half of each site in order to monitor the loss of hydrocarbons. Using various modeling equations, Bragg et al. (1996) determined that the available N per unit oil was the most important factor for removal of easily degraded hydrocarbons. They also noted that more recalcitrant compounds in the oil did not appear to decrease in the nutrient-amended site. Their conclusion, like that of Braddock et al. (1997), was that the optimal level of nutrient amendment was likely to be site specific.

Lai et al. (1996) used a modified shake-flask system to determine the nutrient requirements for naphthenic acid removal from tailings pond water collected at an oil sands plant near Fort McMurray, AB. Radiolabeled model compounds were used to determine the rate and extent of biodegradation. Shake-flasks were modified with a side-arm bearing CO_2 traps in order to measure the extent of mineralization of the model compounds. The dissolved O_2 present in samples was also monitored. Biodegradation of model compounds in tailings pond water was increased by the addition of P over the first 4-5 wk of incubation. After this time, no further stimulation of microbial activity by phosphate was observed, however the authors suggested that the supply of available N may have been depleted at this point (Lai et al., 1996). It is also possible that the accumulation of toxic metabolites may have decreased microbial activity; the authors did not investigate this possibility. For the most part, nutrient amendment investigations had demonstrated that N was the limiting factor for biodegradation, therefore this research was particularly interesting as P was implicated as the limiting factor (Lai et al., 1996). This research further strengthens the argument that augmentation of biodegradation by nutrient amendment is site specific, so that investigation of the requirements of the microbial population at each site is necessary before an effective remediation strategy can be selected.

Mueller et al. (1991) developed a landfarming chamber experimental system to predict the effects of fertilization on biodegradation of pentachlorophenol (PCP) and creosote in contaminated soil and sediment. These authors monitored 42 compounds found in creosote as well as PCP. Compounds were divided into three categories based on their water solubility; these categories can be described using three sample compounds: naphthalene (solubility 30 ppm at 25°C), phenanthrene (solubility 1 ppm at 25°C), and chrysene (solubility 0.1 ppm at 25°C). A surface soil and a subsurface sediment, both contaminated with PCP and creosote, were tested. The concentration of the 42 creosote compounds and PCP, total cultureable heterotrophs and phenanthrene-degrading bacteria were monitored. Samples were amended with medium containing phosphate, NH_4^+ and NO_3^-, or with sterile distilled water. Nutrient supplementation was found to stimulate biodegradation of low solubility compounds in soil and sediment microcosms. However, most of the high molecular weight (mainly the least soluble) contaminants were not degraded significantly in any of the microcosms (Mueller et al., 1991). Nutrient amendment was, however, demonstrated to improve biodegradation of more soluble compounds in surface soils, as well as stimulating the phenanthrene-degrading microbial population to develop early in the incubation period. Interestingly, nutrient amendment did not appear to stimulate biodegradation, or the development of phenanthrene-degrading bacteria, in the subsurface sediment (Mueller et al., 1991). The authors did not discuss this finding.

Piehler and Paerl (1996) examined the potential for enhancing diesel fuel biodegradation with the addition of inorganic nutrients in coastal marine waters. Their research differs from the other investigations discussed here in that the inoculum contained little organic C; as a result these authors found that the addition of particulate organic C greatly stimulated the microbial activity in their samples. Experimental microcosms consisted of 30 mL water that had been chronically exposed to diesel fuel over a period of years, incubated in 60-mL serum bottles, which were shaken to improve aeration and mixing. Dissolved O_2 ranged from 1.6-6.0 mg/L. Rates of biodegradation were highest when both N and P were added to the microcosms, and the authors suggested that N limitation likely occurred first, followed by P limitation as biological activity commenced.

Biodegradation of contaminants in systems inoculated with soil, sediment or contaminated water appears to be governed by a number of factors, including the addition of inorganic nutrients to contaminated soils or water samples. In each of the above cases, either N or P was determined to be the most limiting factor for biodegradation, however in two of these cases (Lai et al., 1996; Piehler and Paerl, 1996), once biodegradation in the presence of one added nutrient commenced, another nutrient soon became limiting. In another case (Braddock et al., 1997) the initial amendment of contaminated materials with both N and P was required to achieve the highest rate of biodegradation. The overlying theme in all the studies was that the nutrient requirements for stimulating microbial activity against contaminant compounds are dependent on the conditions at the contaminated site, and that each site must be assessed before any conclusions regarding its bioremediation can be reached.

1.10 Sulfolane biodegradation

Aerobic biodegradation of sulfolane has been demonstrated by many authors. In almost all cases, a mixed inoculum taken from soils or activated sludge was used to degrade the compound, however Juhl and Clark (1990) and Lee and Clark (1993) both observed activity against sulfolane by a pure culture. Bagnall et al. (1983) filed a patent for a rotary biological contactor used to degrade cyanuric acid. The authors noted that sulfolane can be a co-contaminant with cyanuric acid, as it is used in the manufacture of chlorinated cyanuric acid derivatives. Sulfolane biodegradation commenced after a 6-d lag time. The bioreactor was able to remove up to 3000 mg/L sulfolane when it had an average hydraulic retention time of 66 h. Lower concentrations of sulfolane were completely removed from the effluent with lower hydraulic retention times; 18 h for 2700 mg/L and 12 h for 2400 mg/L.

Two types of bench-scale bioreactor were used by Chou and Swatloski (1983) to test the ability of acclimated or unacclimated sludge to degrade sulfolane. A completely mixed system inoculated with activated sludge was able to remove 80 mg/L sulfolane, with only a few instances of detectable (up to 5 mg/L) levels of sulfolane in the effluent. Reactors were tested with sudden influxes of sulfolane known as 'shock loadings'. In each case a high concentration of sulfolane was introduced to the reactor over a period of up to 5 d. Shock loading of sulfolane in the acclimated reactor resulted in some sulfolane appearing in the effluent. With a shock loading of 300 mg/L, up to 40 mg/L of sulfolane was detectable in the effluent up to 48 h after the loading; by 72 h effluent contained ≤ 3 mg/L sulfolane. A second loading of 300 mg/L sulfolane produced similar results. Different results were obtained with sulfolane shock loading of unacclimated sludge. In this case the addition of 325 mg/L sulfolane resulted in up to 210 mg/L appearing in the effluent for the first 72 h, by 96 h effluent concentrations had decreased to near 40 mg/L and by 120 h to 10 mg/L. Although little or no sulfolane-degrading activity was initially evident, after 5 d of exposure to sulfolane some of the biomass present in the reactor had developed the ability to use this compound.

The continuously stirred tank reactor used by Chou and Swatloski (1983) was able to remove up to 80 mg/L sulfolane, with a hydraulic detention time of 2 d, and sulfolane removal was ≥ 95%. A shock loading of 300 mg/L sulfolane to this system resulted in sulfolane concentrations of up to 16 mg/L in the effluent, with the maximum concentration being observed 15 h after the high sulfolane concentration was introduced. By the second day after the shock loading began, the effluent sulfolane concentration was only 2 mg/L. The authors did not test this system with unacclimated sludge.

During the sulfolane-degrading experiments, Chou and Swatloski, (1983) noted that it was necessary to adjust the pH of the bioreactors because the pH tended to drop as sulfolane was degraded. This was found to be due to acid release during the conversion of the S in sulfolane to $SO_4^{=}$. Potential intermediates of sulfolane degradation were predicted to be butane sulfonic acid, *n*-butanol and butyric acid. The ability of activated sludge from the completely mixed activated sludge system to degrade these compounds was tested in batch cultures. Sulfolane-degrading activated sludge was able to utilize *n*-butanol and butyric acid, however butane sulfonic acid was not removed from the cultures (Chou and Swatloski, 1983), which suggests that butane sulfonic acid may not be an intermediate of sulfolane degradation in this system.

The results of the research carried out by Chou and Swatloski (1983) suggest that sulfolane biodegradation can occur over a long period of time in bioreactors with some recycling of the biomass, such as the continuously stirred tank reactor and the completely mixed activated sludge system. They found that an initial acclimation time was required if the sludge inoculum had not been previously exposed to sulfolane. Both reactors were able to remove sulfolane delivered at initial concentrations of 80 mg/L. Shock loading concentrations of up to four times this amount initially resulted in some sulfolane breakthrough into the reactor effluent, however over a matter of days sulfolane removal from reactors loaded with high concentrations of this compound was possible. Further investigation would be required to determine whether these systems could be used for on-site treatment of sulfolane contamination.

McLeod et al. (1992) tested the ability of activated C columns, some inoculated with biomass, to remove sulfolane from contaminated groundwater. Activated C columns were inoculated with sulfolane-acclimated sludge obtained from a refinery plant. A simplified groundwater substitute was used; this was comprised of tap water containing 1 mg/L sulfolane. In some cases the influent was modified with 10 mg/L sugar to increase biomass. The authors did not specify the type of sugar that was used. Columns were also subjected to air-stripping. Control columns which had no biomass showed no significant decrease in sulfolane in the effluent. Breakthrough of sulfolane in the effluent occurred after 1 wk in columns that were loaded with influent water at 3.0 mL/min and were not fed sugar, 3 wk for a column loaded with influent water at the same rate that was fed 10 mg/L sugar, and 5 wk for a column fed sugar with an influent water loading rate of 1.8 mL/min. None of the three columns was able to absorb significant amounts of sulfolane after 60 d of incubation.

These authors also tested pilot scale biologically activated C columns at the contaminated site, to determine whether the bench scale results could be duplicated. These columns were inoculated with groundwater containing 93-630 μg/L sulfolane over the test period. Additional C sources were the fumigants dibromochloropropane and ethylene dibromide, which were known to be present at the site. The results for the inoculated columns and the uninoculated control column were similar for the pilot scale experiment. The authors attributed these results to the growth of a sulfolane-degrading bacterial community in the control column. This population could have been introduced during the column set-up or from the groundwater. It was estimated that without biodegradation, sulfolane removal from groundwater would have ceased in the columns after 40 d. During the first 2 wk, it was expected that the activated C would absorb most of the sulfolane in the groundwater. Biodegradation was predicted to remove up to 73% of the groundwater sulfolane for up to 40 d. The presence of biocide did not appear to interfere with sulfolane degradation, however McLeod et al., (1992) suggested that this was due to interactions between the biocide and the activated C which protected the bacterial community from the biocide.

The results obtained by McLeod et al. (1992) suggest that activated C columns inoculated with a bacterial population acclimated to sulfolane might be useful for short-term removal of sulfolane from contaminated groundwaters, but these systems appear to become saturated over a relatively short period of time.

Lee and Clark (1993) discovered a *Pseudomonas maltophila* isolate able to degrade aromatic sulfonic acids. The authors tested the ability of this organism to metabolize other substrates, and determined that it was able to grow on minimal agar supplemented with 1 g/L sulfolane as a carbon source. Juhl and Clark (1990) developed a series of *Escherichia coli* mutants that were capable of removing thiophene from liquid culture. The thiophene-degrading mutants demonstrated a sulfone oxidase activity that was also stimulated by sulfolane; these mutants were able to utilize sulfolane on tetrazolium agar indicator plates (Bochner and Savageau, 1977).

More recently, Fedorak and Coy (1996) demonstrated the removal of sulfolane from laboratory shake-flask and soil column (landfarming chamber) microcosms inoculated with soils, groundwaters and/or sandstone from a sulfolane-contaminated aquifer and incubated aerobically. Aquifer slurry shake-flask cultures containing sandstone and groundwater were able to biodegrade sulfolane when incubated at 26°C or at 8°C. Incubation at the higher temperature increased the biodegradation rate (6-8 mg/L/day and 4 mg/L/d respectively, for N- and P-supplemented cultures). Supplementation with N and P also increased the biodegradation rate of these cultures; supplemented cultures at 8°C removed sulfolane at 4 mg/L/d while unsupplemented cultures removed sulfolane at 0.8-1 mg/L/d. The authors also demonstrated that cultures inoculated with groundwater but no soil or sandstone were able to degrade sulfolane, although this activity was much slower than that observed for samples inoculated with solid materials. This result is similar to the findings of Holm et al. (1992): that groundwater microbial populations were able to remove the same amount of a contaminant compound as aquifer sediment populations, but at a much lower rate.

The soil columns utilized by Fedorak and Coy (1996) were based on the landfarming chamber design of Mueller et al. (1991). Soil column microcosms inoculated with previously sulfolane-contaminated soil and bedrock material were able to remove sulfolane at 0.5 mg/L/d in a N- and P-supplemented microcosm, and at 0.4 mg/L/d for an unsupplemented microcosm (Fedorak and Coy, 1996). Microcosms inoculated with material taken from a new well area ('till') demonstrated a lag period of 15 d for N- and P-supplemented 'till' material; the microcosm containing unsupplemented 'till' material had a 22 d lag period. After the initial lag periods, the microcosms removed sulfolane at 0.7 mg/L/d and 0.5 to 0.8 mg/L/d, respectively.

Fedorak and Coy (1996) suggest that bioreactors or bioventing might be useful means of intrinsic bioremediation of sulfolane-contaminated sites. Their work also demonstrated that sulfolane-degrading populations exist at contaminated sites, and that supplementation with N and P can increase the rate of sulfolane biodegradation and decrease the lag time before degradation commences.

In contrast with the previous authors, Bridié et al. (1979) determined that an inoculum from a biological sanitary waste treatment plant was unable to degrade sulfolane over a period of 5 d. These authors determined the theoretical O_2 demand for sulfolane oxidation to CO_2, water and sulfuric acid to be 1.73 g O_2 per 1 g sulfolane, the chemical O_2 demand to be 1.75 g O_2 per 1 g sulfolane (101% of the theoretical O_2 demand) and the biological O_2 demand to be 0 g O_2 per 1 g sulfolane. It is likely that the amount of time allowed for acclimation of the biological sanitary waste inoculum to sulfolane was not sufficient for the onset of biodegradation to occur in the experiments by Bridié et al. (1979). Bagnall et al. (1983) observed a 6-d lag period before sulfolane commenced in unacclimated sludge, and Chou and Swatloski (1983) saw a 5-d lag time before sulfolane degradation began in an unacclimated sludge from a sewage treatment plant.

1.11 The relevance of laboratory biodegradation experiments to microbial activity in the field

Laboratory experiments are important for predicting the possibility of bioremediation in the field. Although laboratory treatments such as shake-flask or slurry cultures can often overestimate the potential for biodegradation of contaminant compounds, they can be used successfully to detect organisms capable of degrading the compounds of interest. Laboratory experiments have the advantage of being quick, simple, and easy to control (Holm et al., 1992). Furthermore, laboratory experiments can be used to predict the nutrient modifications that might be required at a given site in order to stimulate microbial activity, and to determine the conditions under which the population can best remove the compounds of interest (Ritter and Scarborough, 1995). Experiments that link laboratory studies with *in situ* activity have been carried out (Holm et al., 1992; Nielsen et al., 1995a; 1995b; 1996). Nielsen and Christensen (1994) also tested samples taken from a number of sites in a contaminated aquifer to determine whether significant variability occurred within a contaminated region, and Fu et al. (1996) evaluated the differences between different types of laboratory microcosms for predicting the biodegradation rates of some compounds.

Holm et al. (1992) carried out a series of experiments to determine whether bacteria attached to sediment particles or those found in groundwater were responsible for degradation of xenobiotic organic contaminants in an aerobic aquifer located at a landfill in Jutland, Denmark. These experiments were done in laboratory microcosms, which replicated *in situ* microcosms installed in the contaminated aquifer. The authors determined that most microbial activity was connected with the sediment present at the site. However, they also found that the microbial community of groundwater alone could remove the same amount of contaminant compounds as groundwater- and sediment-containing microcosms, although the rate of degradation was lower. Experiments involving sediment suspensions yielded similar results in the laboratory and the field. It was determined that the majority of bacterial activity was associated with the fine (silt- and clay-sized) sediment particles, as microcosms containing only these particles demonstrated a similar level activity to microcosms containing entire sediment samples. It was deemed to be advantageous to use only the fine sediment particles in laboratory experiments, since larger particles could seriously complicate sampling procedures. Groundwater-containing experiments provided higher degradation rates in the field than in the laboratory, however the field containment device had a great deal more surface area than the laboratory incubation bottles, which may have contributed to the degradation rate (Holm et al., 1992). The increased surface area likely provided a solid support for attachment of active microorganisms.

Nielsen et al. (1995a; 1995b; 1996) carried out a series of experiments at the same landfill site as Holm et al. (1992). In the anaerobic portion of the landfill leachate plume, phenolic compounds were found to be transformed or degraded more frequently *in situ* than in the laboratory, possibly due to the presence of Fe(III) as a terminal electron acceptor in some of the *in situ* microcosms which was absent in the laboratory microcosms (Nielsen et al., 1995a). In the same anaerobic portion of the landfill leachate plume, Nielsen et al., (1995b) found very similar results for transformation of aromatic and chlorinated aliphatic compounds *in situ* and in the laboratory, although more compounds were transformed in the *in situ* microcosms than the laboratory microcosms. The aerobic aquifer study carried out by Nielsen et al. (1996) utilized a pristine, shallow aquifer close to the landfill site used in previous studies. In this aquifer, field and laboratory studies again demonstrated similar results. The experiments of Holm et al. (1992) and Nielsen et al. (1995a; 1995b; 1996) demonstrate that laboratory microcosm studies can be used to predict biological activity against xenobiotic compounds in the field. In some cases *in situ* activity was underestimated in the laboratory studies, possibly due to the greater variety of nutrients available in an aquifer, but on the whole laboratory studies were judged to be useful for predicting microbial activity at contaminated sites.

Experiments done by Nielsen and Christensen (1994) demonstrated that samples taken from different sites at the same aquifer had a similar ability to degrade aromatic hydrocarbons, provided that they were taken from portions of the aquifer with similar hydrogeology. In total, eight localities representing a 15 x 30 m section of an aerobic aquifer were tested. Microcosms were incubated in 2.5-L glass bottles, and the groundwater portion of each sample was saturated with O_2 by bubbling with air before the experiments began. Biodegradation of test compounds (seven different aromatic compounds) at concentrations of up to 150 μg/mL did not decrease the concentration of O_2 below 9 mg/L over a 149-d incubation period. Samples taken from parts of the aquifer that had a slightly different source of groundwater displayed some differences in their ability to degrade certain compounds. Samples taken from different parts of the aquifer that shared the same groundwater source generally demonstrated similar biodegradative abilities (Nielsen and Christensen, 1994).

The type of laboratory microcosm used in an experiment can also have an effect on the predictions made about biodegradation in an aquifer. Fu et al., (1996) compared biodegradation rates for phenol in slurry, wafer and compacted soil tube reactors. Slurry reactors resembled aerobic shake-flask experiments, and were the least representative of the conditions found in an aquifer. Rates of biodegradation in these reactors were much faster than in the other reactors. Soil wafers most closely imitated bioventing or landfarming techniques, where O_2 limitation in the soil is minimized. The compacted soil tube reactors depended on diffusion of nutrients and O_2 into the soil sample, and most closely resembled *in situ* bioremediation. Degradation rates in the compacted soil tube reactors were the slowest found in the three laboratory microcosm types. Biodegradation of phenol was monitored partly using O_2 uptake rates in the different reactors. O_2 uptake rates for the reactors were found to be 6 mg/L/h for the slurry reactor and 0.8 mg/L/h for both other reactors when soil was loaded with 7.5 mg phenol. With a phenol load of 3.75 mg, O_2 uptake rates were 2.9 mg/L/h in the slurry reactor, 0.38 mg/L/h in the wafer reactor, and 0.3 mg/L/h in the compacted soil tube reactor. Overall, the authors found that as the complexity of the system increased, the biodegradation rate decreased; for instance the additional factor of diffusion of nutrients such as O_2 slowed biodegradation when compared with a system that was not dependent on diffusion (Fu et al., 1996).

1.12 Principal points addressed in this thesis

The research done for this thesis project was divided into two parts. The first part was an assessment of the ability of microorganisms present in aquifer sediments and groundwaters, some contaminated with sulfolane, to degrade this compound. The second portion of the research focused on an investigation of some properties of sulfolane biodegradation and the bacteria involved in this activity, using mixed and pure enrichment cultures of sulfolane-degrading bacteria.

The first two site assessment projects were initiated by the Canadian Association of Petroleum Producers (CAPP). This organization wished to demonstrate the presence of sulfolane- and DIPA-degrading organisms at contaminated sites in western Canada. The first project was to determine the rate and extent of sulfolane and DIPA biodegradation under both aerobic and anaerobic conditions, using materials from contaminated aquifers as a source of microorganisms. The second project undertook to examine the biodegradation rate constants for sulfolane and DIPA under different conditions: low aeration and mixing (2.5-L microcosms), low but constant dissolved O_2 (respirometer microcosms), and in shake-flask cultures supplied with various nutrients. The sulfolane degradation portion of this research was part of this thesis project. Due to time and financial restraints, the scope of these projects was mainly limited to the requirements of CAPP. The third site assessment project was not initiated by CAPP;

however, materials used for inocula were provided by CAPP. The purpose of this project was to monitor the sulfolane-degrading population of a contaminated subsurface sample during sulfolane degradation, using a laboratory system that mimicked a biopile remediation system (Blackburn and Hafker, 1993; Fedorak and Coy, 1996; Mueller et al., 1991). The relationship between microbial activity in shake-flask cultures and in more complex microcosms was also explored. Overall, the purpose of this series of research projects was to determine whether sulfolane biodegradation could be stimulated in the field, based on laboratory evaluations of the microbial populations present at contaminated sites and their nutrient requirements.

In order to fully understand the biodegradation of a chemical, it is important to understand each step in the catabolic process by which it is removed from a contaminated environment (Bouwer, 1992; Sims et al., 1990). The second portion of this thesis project was directed toward obtaining information on sulfolane mineralization by mixed and pure enrichment cultures, identification of one or more bacterial species involved in this activity, and possibly identification of products of sulfolane degradation other than CO_2 and $SO_4^=$. These metabolites were predicted to be small ($\leq C_4$) and highly polar, therefore were predicted to be difficult to detect. Different methods for detection of these compounds were attempted, but none was successful, therefore the second half of this thesis project was focused toward obtaining accurate C and S mass balances for sulfolane degradation by different cultures, and the identification of the most active pure sulfolane-degrading bacterial culture. The pure and mixed cultures used in these experiments were all isolated from the three sulfolane-contaminated sites studied in the microcosm experiments. Thus, there is a potential that with the addition of the appropriate nutrients, the sulfolane degradation demonstrated in the mineralization experiments could be stimulated on site.

The objectives and organization of this thesis can be summarized as follows:

A) Site oriented research
   1) Assessment of the potential for aerobic and anaerobic sulfolane biodegradation in contaminated aquifer sediment from three Sulfinol® plant sites.
   2) Assessment of the nutrient requirements for aerobic sulfolane biodegradation in aquifer sediment and groundwater or wetland materials and surface water taken from one of the contaminated sites described in objective A1.
   3) Development of a medium for detecting and enumerating of sulfolane-degrading microorganisms.

4) Assessment of sulfolane biodegradation in a laboratory scale air sparged microcosm ('biopile') experiment, which could be used to make predictions regarding on-site bioremediation strategies.

B) Determination of the characteristics of sulfolane biodegradation by pure and mixed bacterial cultures in the laboratory.

1) Measurement of the extent of sulfolane mineralization to CO_2 and $SO_4^{=}$ and conversion to biomass in mixed and pure bacterial cultures.

2) Identification of a sulfolane-degrading bacterium to the genus level.

2 MATERIALS AND METHODS

2.1 Soil and groundwater sampling and description of samples

Three projects were done using soil, aquifer sediment, and groundwater samples taken from three sour gas treatment plant sites. Materials from sour gas treatment plant sites were provided by the CAPP through Komex International, Ltd. (Calgary, AB). Uncontaminated soils used in these experiments were obtained from the University of Alberta or from commercial sources. The soil and groundwater characteristics are summarized in Tables 2.1 through 2.4. The soils and sampling sites are described in Appendix A. A map of site 2 is shown in Figure 2.1. The experiments in which these samples were used are described in Sections 2.6 to 2.8.

2.2 Analytical techniques

2.2.1 Gas chromatography

2.2.1.1 Sulfolane analysis

Three methods were used to analyze sulfolane by gas chromatography (GC). Initially, sulfolane was extracted into dichloromethane, using dibenzothiophene as an internal standard, and analyzed on a Hewlett-Packard (HP) model 5730 GC equipped with a flame ionization detector (FID) and a 0.25 mm by 25 m (0.25 μM thickness) DB5 column (J&W Scientific, Inc.) with a split ratio of 20:1. The injector and detector were at 250°C. The oven temperature program was 90°C for 2 min, 4°C/min to 250°C, which was held for 16 min. Sample injections were 2 μL. The sample peaks were measured and recorded using a HP model 3390A integrator. This method was originally described by Fedorak and Coy (1996).

The second method for sulfolane analysis employed a direct aqueous injection method originally developed for analysis of martian surface samples (Novotny et al, 1975) that was later used for analysis of phenol and similar compounds (Bartle and Elstub, 1977; Budwill et al., 1996). A 2 m x 2.8 mm stainless steel column packed with Tenax-GC (Alltech Associates, Deerfield, IL) coated with 5% polyphenyl ether (6 ring) was used in either a HP model 5700A or a model 5890 GC, equipped with a FID. Columns were prepared as described in Bartle and Elstub (1977). Tenax® GC is no longer available; Alltech Associates now provides a similar material called Tenax® TA. Injector and detector temperatures were 250°C, the oven temperature was isothermal at 210°C.

Table 2.1. Characteristics of aquifer sediment samples taken from sites 1, 2 and 3.

site	sulfolane contamination	groundwater sulfolane concentration (mg/L)	groundwater DIPA concentration[a] (mg/L)	characteristics of sediment used in the microcosms[b]
1	contaminated	4	< 1	crushed sandstone bedrock
2	contaminated	310	< 1	clay rich glacial till
2	uncontaminated	< 1	< 1	silty clay till
3	contaminated	30	6	fine to medium grain sand

[a] Detection limit 1 mg/L (Gieg et al., 1998).

[b] Soil samples are described by Luther et al., (1998).

Table 2.2. Description of aquifer sediment and wetlands materials from the site 2 locations.[a]

sample	sulfolane in groundwater (mg/L)	total organic C from sulfolane (mg/L)	total organic C in water sample (mg/L)	soil lithology[b]
east plume aquifer material	493	197	650	silty clay and silty sand
west plume aquifer material	675	270	550	silty clay
uncontaminated aquifer material	<1	<1	<1	sandy silt
west wetlands material	<1	<1	ND[c]	clay loam
east wetlands material	<1	<1	ND[c]	loam sand

[a] The sampling locations are shown in Figure 2.1.

[b] Soil lithology information was provided by J.H. Sevigny, Komex International Ltd., Calgary AB.

[c] ND not measured

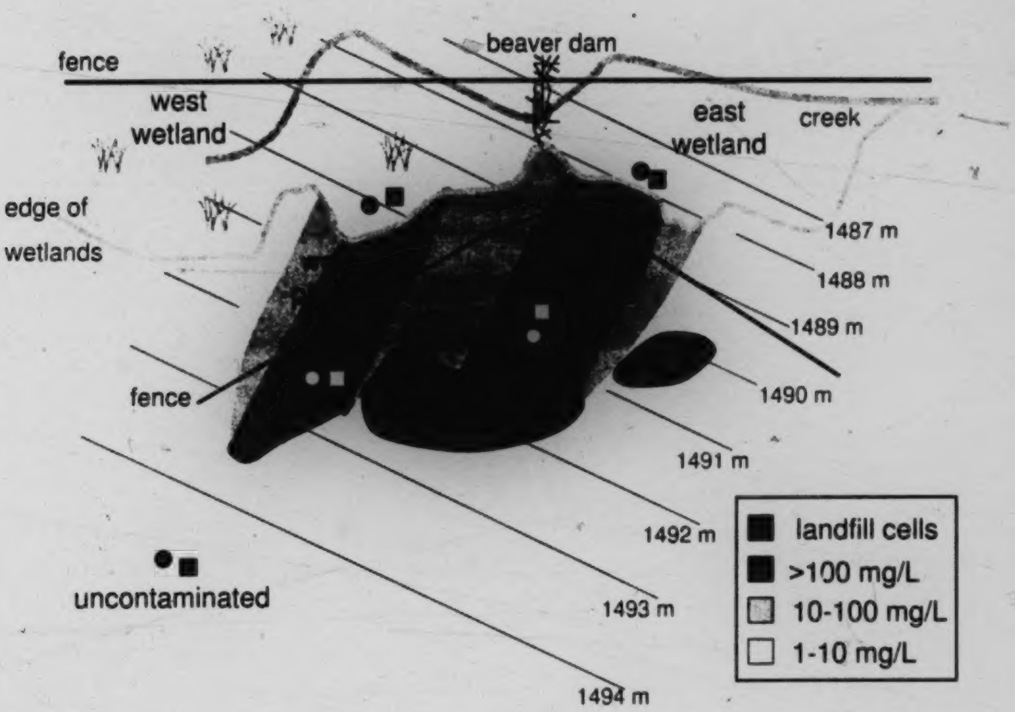


Figure 2.1. Site 2 locations from which samples were taken for population and nutrient screening experiments. The sulfolane contamination in different areas is marked with varying shades of grey. The elevation of the site is marked by lines, labeled in m. Groundwater flow is toward the weltand regions, along the downward slope of the land. Locations of aquifer sediment or wetland mud sampling are marked with a circle; locations from which the corresponding water samples were taken from piezometers or surface water are marked with a square. Information and site maps were provided by J. H. Sevigny (Komex International, Ltd., Calgary, AB).

Table 2.3. Total C, N and P content of soils and aquifer sediments used in the nutrient amendment experiments.

soil or sediment sample	% C in soil	% N in soil	% P in soil
east plume	2.42	0.08	0.06
west plume	1.84	0.09	0.06
uncontaminated	1.68	0.07	0.06
Sulfinol® plant #1	1.37	0.07	0.06
Sulfinol® plant #2	2.23	0.08	0.04
topsoil	23.1	1.30	0.13

Table 2.4. Characteristics of site 2 sulfolane-contaminated sediment taken from near two Sulfinol® process buildings.[a]

sample	sulfolane in sediment (mg/L)[b]	DIPA in sediment (mg/L)[b]	description of sediment
Sulfinol® #1	30	< 0.5	sandy clay, gravelly-cobbly till, 35 to 60 % coarse fragments
Sulfinol® #2	43	1	sandy clay till, 5 % coarse fragments, strong Sulfinol® odor

[a] These samples were used for air-sparged microcosm experiments.

[b] Information was provided by B. Samis, Komex International Ltd., Calgary, AB.

The column was preconditioned as suggested by Supelco (Sigma-Aldrich Canada, Oakville, ON): purge with carrier gas for 10 min at room temperature, heat to 300°C for 2 h and cool. Further preconditioning was then carried out: the column was injected with 2 μL of distilled water until no peaks could be detected, then injected with 2 μL 500 mg/L sulfolane in distilled water until the peak height stabilized, then with distilled water until residual sulfolane peaks disappeared. After preconditioning, sulfolane peak heights were generally reproducible, with a standard deviation of <1%.

Sample peaks were measured using a HP model 3390A integrator. The detection limit for sulfolane using this method was 1 mg/L on the model 5700A GC and 0.5 mg/L on the model 5890 GC, when 2 μL samples were injected. Sulfolane peaks appeared at approximately 12 min on the 5700A GC and 11 min on the 5800 GC. Derivatives of sulfolane, including sulfolene, 3-methylsulfolane, 3-hydroxysulfolane, and 2,4-dimethylsulfolane were also analyzed by this GC method, except that the oven temperature was kept isothermal at 80°C for sulfolene.

The direct aqueous injection method was later modified by altering the column length to 1.3 m, with a 15-cm length of packed column added to the front end of the analytical column with a steel fitting, as a guard column. The guard column was developed because it was determined that most column deterioration was the result of debris building up in the first 2 to 5 cm of the column. Replacing the guard column required a smaller amount of time and materials than cleaning or replacing the analytical column. Preconditioning of the guard column was carried out in a similar manner to preconditioning of the entire column. Samples of 2 μL were injected into the column. Sulfolane peaks eluted between 4 and 5 min. The oven temperature was 200°C for sulfolane, 3-hydroxysulfolane, 3-methylsulfolane, 2,4-dimethylsulfolane, butane sultone, and tetrahydrothiophene sulfoxide. Sulfolene and thiolane were analyzed at 50°C and 1-butanesulfonic acid was analyzed at 60°C using this GC method.

The third method for sulfolane analysis utilized a HP 5890 GC equipped with a FID, a flame photometric detector (FPD) and a 0.25 mm by 25 m (0.25 μM thickness) DB5 column (J&W Scientific, Inc.). Samples of 2 μL, dissolved in dichloromethane or ethyl acetate, were injected by hand or using an automated HP 7673 GC/SFC autoinjector sampling system. Gas flows were 25 mL/min H_2, 260 mL/min air and 0.9 mL/min He carrier gas. The injector temperature was 260°C, the FID temperature was 270°C and the FPD temperature was 250°C. The standard oven temperature program for this GC was 90°C for 2 min, 4°C/min to 250°C, where the temperature was held for 18 min. A second temperature program was designed to separate the solvent peak from potential early-eluting sample peaks: this program was 60°C for 2 min, 4°C/min to 120°C, 10°C/min to 250°C, where the temperature was held for 29 min. A two-channel HP 5880 GC integrator was connected to record the peaks from the two detectors.

2.2.1.2 Acetate analysis

Acetate analysis was measured by GC as described by Boone (1982) and Gieg (1996). Samples of 2 μL were injected into a HP model 5700 GC equipped with a FID and a glass column (2 m x 2 mm) packed with GP 10% SP 1000/1% H_3PO_4 on 100/200 Chromosorb WAW (Supelco, Sigma-Aldrich Canada, Oakville, ON). The injector and detector were set at 225°C and 250°C, respectively. The oven temperature was 120°C. Gas flows were 60 mL/min for the carrier gas, N_2; the air and H_2 flow rates were 250 mL/min and 60 mL/min, respectively. The sample peaks were measured and recorded using a HP model 3390A integrator. For all samples and standard solutions, 10 μL of 4 M H_3PO_4 was added to 90 μL of aqueous sample prior to analysis.

2.2.1.3 Methane analysis

Methane was analyzed by injecting 100 μL samples of headspace gas into a HP model 5700A GC equipped with a 2 m x 2.8 mm stainless steel column packed with Tenax-GC (Alltech Associates, Deerfield, IL) coated with 5% polyphenyl ether (6 ring) and a FID. This column was also used for sulfolane analyses; the operating conditions for methane analyses were similar except that the oven was kept at room temperature. Methane standards were prepared as described by Nelson (1971). The sample peaks were measured and recorded using a HP model 3390A integrator.

2.2.1.4 CO_2 and N_2O analysis

Detection of N_2O was achieved using a Varian Aerograph model 700 GC equipped with a 3 m x 0.5 cm column packed with Poropak R and a thermal conductivity detector (TCD). The detector was operated at 90°C and 150 mV. The injector was at room temperature (25°C) and the oven temperature was 60°C. Helium was used as a carrier gas. This method was described by Budwill et al. (1996). Some experiments requiring CO_2 quantification were carried out using this equipment at the same settings.

Quantification of CO_2 was also done using a HP model 5890 Series SII GC equipped with a TCD and a 2 m x 2.8 mm stainless steel column packed with Poropak

R. The carrier gas was He at a flow rate of 58 mL/min. Peak areas for both GCs were measured using an HP3390A integrator.

2.2.3 UV/VIS spectrophotometry

UV/VIS spectrophotometry was done using a Philips Model PU 8740 UV/VIS scanning spectrophotometer. The absorbance spectrum for sulfolane and DNA quantification were done using a Unicam UV3 UV/VIS Spectrometer.

2.2.4 Measurement of pH

The pH of various solutions was measured using a Fisher Accumet pH meter equipped with a probe obtained from Parmer (Chicago, IL). Some solutions were measured using colorpHast pH paper strips obtained from EM Science (Gibbstown, NJ).

2.2.5 Atomic Absorption spectroscopy

Atomic absorption spectroscopy was used to measure Mn(II) and total extractable manganese in samples. Analyses were done using a Perkin-Elmer 3300 Atomic Absorption Spectrophotometer equipped with a Mn lamp. Absorbance was measured at 279.5 nm. An IBM model 486 computer with Perkin-Elmer software was used for data analysis.

2.2.6 Ion chromatography

Ion chromatography to measure $SO_4^=$ was done using a Dionex Series 2000i/SP ion chromatograph equipped with a Dionex IonPac AS4A 2 x 250 mm anion exchange column (Dionex, Sunnyvale, CA) and a guard column (specified by the manufacturer). The output range for the ion chromatograph was 30 µS and the sensitivity range was set to 0 to 20 mg/L. Samples were filtered with a CoStar µStar 0.22 µm pore size filter before analysis, and diluted with filtered, deionized, distilled water when necessary.

2.2.7 Total organic carbon analysis

Total organic C analyses were carried out using an Ionics model 1505 Programmable Carbon Analyzer (Ionics, Inc.). The oven was set at 850°C, and the purgeable organic C (POC) program was used set to a range of 10 to 50 mg/L C. Samples were diluted with deionized, distilled water if the total organic C concentration was above this range.

2.2.8 Dissolved oxygen analysis

Dissolved O_2 was measured using a YSI Model 59 O_2 meter. For each sample, the probe was immersed in the shaking culture for 1 to 2 min before the dissolved O_2 reading was taken. The probe was disinfected with 95% ethanol between each sample.

2.2.9 Fluorometry

Fluorometry was done using a Hoefer Scientific Instruments Model TKO 100 fluorometer. DNA sample concentrations were estimated by agarose gel electrophoresis and comparison with standard DNA samples; DNA samples for fluorometry were then prepared at approximately the appropriate concentration for sequencing or PCR reactions. Samples of 2 µL were mixed with 1 mL Hoefer Scientific H33258 fluorometry dye, used according to the specifications of the manufacturer, and analyzed.

2.2.10 Chemical analytical techniques

2.2.10.1 Iron (II) and total iron analyses

Ferrous iron [Fe(II)] was analyzed using the method of Lovley and Phillips (1986). This method makes use of the reaction between divalent cations such as Fe(II) and ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,3,5-triazine], which forms a water-soluble magenta complex that can be quantified spectrophotometrically at 520 nm (Stookey, 1970).

Standards for this assay were prepared using a 2 g/L Fe(II) stock solution generated by dissolving 1.404 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in 20 mL H_2SO_4 and deionized, distilled water, made up to a total volume of 100 mL. The calibration curve for the assay ranged from 0 to 500 mg/L Fe(II). Standards were added to 4.9 mL 0.5 M HCl and treated as samples for analysis.

Samples were analyzed by placing 0.1 mL of sample slurry (containing resuspended soil and supernatant from anaerobic microcosms) in 4.9 mL of 0.5 M HCl, to extract the Fe(II) from the soil (Chao and Zhou, 1983). Slurry samples (0.1 mL volume) for analysis of total extractable iron were placed in 4.9 mL 0.25 M hydroxylamine hydrochloride, following the method of Lovley and Phillips (1988), based on the extraction technique described by Chao and Zhou (1983). This solution extracts both Fe(II) and Fe(III) from soil.

After addition of sample or standard solution to the extraction tubes, samples were mixed by vortex and incubated for 15 min, then 0.1 mL of extracted sample was added to 4.9 mL of 1 g/L ferrozine in 50 mM HEPES (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid, pH 7) in glass test tubes. These samples were mixed by vortex and quantified by measuring absorbance at 562 nm.

2.2.10.2 Mn(II) and total extractable Mn preparation for analysis

Manganese(II) or Mn(II) and Mn(IV) together were extracted in the same manner as Fe(II) or Fe(II) and Fe(III), as described above and by Lovley and Phillips (1988).

However, after the extraction with 0.5 M HCl or with 0.25 M hydroxylamine hydrochloride, Mn samples were spun at maximum speed in a clinical centrifuge (approximately 890 x g) for 10 min, then supernatants were diluted in deionized, distilled water to yield a range of 0 to 8 mg/L Mn in a final volume of 2 or 2.5 mL.

Manganese standards were prepared from a stock solution of 995 mg/L Mn metal in 0.3 M HNO_3 (Baker Chemical Co., Phillipsburg, NJ). Analysis of standards generated a straight line calibration curve between 0 and 8 mg/L Mn. Dilutions of standards were prepared immediately before analyses were done.

Standards and samples were analyzed by atomic absorption spectroscopy (described in Section 2.2.5). The autoinjector used a wash solution of 1.5 mL HNO_3 per 1 L deionized, distilled water to rinse between sample analyses.

2.2.10.3. Nitrate analysis

The method used for NO_3^- analysis was based on a colorimetric assay devised by Cataldo et al. (1975). The original assay is based on the nitration of salicylic acid under acidic conditions, which causes the formation of a compound that is yellow at pH ≥ 12, and can be quantified by measuring the absorbance of the assay solution at 410 nm. The assay method of Cataldo et al. (1975) required a sample volume of 25 mL. Due to the small size of the aquifer sediment microcosms and the large number of samples processed in this work, it was more practical to work with a sample volume of 100 µL and a final volume of 10 mL.

A NO_3^- stock solution was made by dissolving 1.63 g KNO_3 in 1 L deionized, distilled water. Standards containing 1 to 60 µg NO_3^- per 0.1 mL were made. For sample analysis, soil slurries removed from anaerobic microcosms were centrifuged at 14 000 rpm in an Eppendorf microfuge for 10 min, then 0.1-mL samples were removed from each tube and placed in glass test tubes. Samples were diluted in deionized, distilled water before analysis when necessary.

The 0.1-mL samples or standards were mixed with 0.4 mL 5% w/v salicylic acid in concentrated H_2SO_4 and allowed to react at room temperature for 20 min, then 9.5 mL 2 M NaOH was added to each reaction mixture. The samples were cooled to room temperature, then the absorbance at 410 nm was measured by spectrophotometry. The method gave a calibration curve linear to 300 mg/L NO_3^-.

2.2.10.4 Turbidimetric sulfate analysis

Sulfate analysis was done by ion chromatography (described above), or by the turbidimetric method described in Standard Methods (1989), modified to suit a 25-mL sample size. Standards were made from a stock solution containing 1.479 g Na_2SO_4 per 1 L deionized, distilled water. The calibration curve was linear within a range of 0 to 280 mg/L $SO_4^=$. Samples with $SO_4^=$ concentrations outside this range were diluted with deionized, distilled water before analysis. Samples were prepared by pelleting bacterial cells with a centrifuge, then filtering the supernatant with a CoStar µStar LB 0.22 µm pore size CA filter to remove other particulate matter, then were refrigerated at 4°C until analysis. Phosphate interferes with the turbidimetric method of $SO_4^=$ analysis, therefore little phosphate could be added to medium used to generate these samples.

A 25-mL sample of filtered culture supernatant or standard was mixed with 5 mL 'Buffer A': 30 g $MgCl_2 \cdot 6H_2O$, 5 g CH_3COONa, 1 g KNO_3, 20 mL 99% CH_3COOH per 1 L deionized, distilled water (Standard Methods, 1989). Samples were stirred continuously while 1.5 to 2.0 g $BaCl_2$ was added. Exactly 1 min after $BaCl_2$ was added, a 1-mL sample was removed and the OD at 420 nm was measured. $BaSO_4$ precipitate forms when $BaCl_2$ is added to a $SO_4^=$-containing solution in the presence of 16.5% (v/v) 'Buffer A' (Standard Methods, 1989), and can be quantified in this manner.

2.2.10.5 Sulfide analysis

Sulfide was analyzed using the methylene blue sulfide assay (Standard Methods, 1989). A stock solution of sulfide was prepared by dissolving 0.75 g $Na_2S \cdot 9H_2O$ crystals (water-washed and dried) in anoxic 0.01 M NaOH in an N_2 atmosphere. This stock solution was stored in a 158-mL serum bottle capped with a butyl rubber stopper and crimped aluminum seal. The actual sulfide concentration in the stock solution, 347 mg/L, was determined by iodometric titration (Standard Methods, 1989).

Samples of 100 µL of the supernatant from soil slurry sample, or sulfide stock solution ranging from 1 to 6 µg (0 to 17 µL) sulfide, were reacted with 4.9 mL DMPD reagent (1.0 g N,N-dimethyl-*p*-phenylenediamine·HCl, 1.0 g $Zn(CH_3COO)_2 \cdot 2H_2O$ and 10 mL concentrated H_2SO_4 per 1 L deionized, distilled water), 5 mL deionized, distilled water and 100 µL of ferric reagent (2.5 g $FeCl_3 \cdot 6H_2O$ per 10 mL deionized, distilled water). The resulting solution was analyzed by measuring absorbance at 660 nm. Poor results were obtained for the soil slurry supernatants, therefore an extraction step was added. Soil slurry samples (100 µL) were extracted in 2 mL DMPD reagent made with 50 mL concentrated H_2SO_4, to release sulfide that was associated with soil or had precipitated with iron in the microcosms. After a 30 min incubation time, 1 mL of extraction supernatant was reacted with 5 mL DMPD reagent, 4 mL deionized, distilled water and 100 µL ferric reagent. With this method, qualitative or semi-quantitative sulfide detection was possible. This method was devised by L.M. Gieg (Fedorak et al., 1997).

Sulfide analysis for the 2.5-L microcosms was carried out by M. Hicks (Dept. of Biological Sciences, University of Alberta, Edmonton, AB) using the method of Ulrich et al. (1997). Sediment samples were removed from the settled sediment region of the three 2.5-L microcosms inoculated with contaminated or uncontaminated aquifer sediments, place in sterile bottles, and immediately transported into an anaerobic chamber with an atmosphere of 5% H_2, 10% CO_2 and 85% N_2, where 1 g of each sample was transferred to each serum bottle used for the passive extraction technique.

2.3 Media

2.3.1 Media for aerobic incubations

B+NP medium contains 2.0 g Na_2SO_4, 0.2 g $MgSO_4 \cdot H_2O$, 2.0 g KNO_3, 1.0 g NH_4Cl, trace amounts of $FeSO_4 \cdot 7H_2O$ and 1.0 mL trace metals solution per 1 L deionized, distilled water. Trace metals solution contains 3.7 g $CaCl_2 \cdot 2H_2O$, 2.5 g H_3BO_3, 0.65 g $FeCl_3$, 0.01 g $CoCl_2$, 0.44 g $ZnCl_2$, 0.87 g $MnCl_2$, 0.29 g $Na_2MoO_2 \cdot 2H_2O$ and 0.0001 g $CuCl_2$ per 1 L deionized distilled water (Fedorak and Grbić-Galić, 1991).

B+N8P medium was described by Kropp et al. (1994). It contains 2.0 g Na_2SO_4, 1.0 g KNO_3, 2.0 g NH_4Cl, trace amounts of $FeSO_4 \cdot 7H_2O$, 100 mL phosphate stock, 5.0 mL $MgSO_4$ stock and 1.0 mL trace metals solution per 1 L deionized, distilled water. Phosphate stock contains 16 g KH_2PO_4 and 24 g K_2HPO_4 per 1 L deionized, distilled water and $MgSO_4$ stock contains 40 g $MgSO_4 \cdot H_2O$ per 1 L deionized, distilled water (Kropp et al., 1994). Phosphate and $MgSO_4$ stocks were autoclaved separately and added to B+N8P after sterilization.

B+NP (low N) medium was developed by Gieg et al. (1998). This medium is a modified version of B+NP medium which consisted of 2.0 g Na_2SO_4, 0.2 g $MgSO_4 \cdot H_2O$, 0.1 g KNO_3, 0.06 g NH_4Cl, 2.0 g K_2HPO_4, 1.0 mL trace metals solution and trace amounts of $FeSO_4 \cdot H_2O$ per 1 L deionized, distilled water.

Sulfate-free medium (Fedorak and Coy, 1996) contained 0.5 g K_2HPO_4, 2.0 g NaCl, 0.2 g $MgCl_2$, 2.0 g KNO_3, 1.0 g NH_4Cl, trace amounts of $FeCl_2 \cdot H_2O$ and 1.0 mL trace metals solution (Fedorak and Grbić-Galić, 1991) per 1 L deionized, distilled water.

2.3.2 Media for anaerobic incubations

Media for anaerobic incubations were prepared under an atmosphere of 30% CO_2, 70% N_2. All the ingredients of the media except the vitamin and mineral solutions and $NaHCO_3$ were combined in water, then the solution was boiled while bubbling with

30% CO_2, 70% N_2. Once the solution reached boiling temperature, it was cooled on ice to room temperature, after which the vitamin and mineral solutions and $NaHCO_3$ were added. Media were then dispensed into serum bottles flushed with 30% CO_2, 70% N_2, which were sealed with butyl rubber stoppers and crimped with aluminum caps before autoclaving.

Medium for NO_3^- reduction contained 0.5 g K_2HPO_4, 2.0 g Na_2SO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 2.0 g KNO_3, 0.06 g NH_4Cl, trace amounts of $FeSO_4 \cdot 7H_2O$, 1.0 mL Wolfe's vitamin solution and 2.5 g $NaHCO_3$ per 1 L deionized, distilled water.

Wolfe's vitamin solution (ATCC, 1992) contained 2.0 mg biotin, 2.0 mg folic acid, 10.0 mg pyroxidine-HCl, 5.0 mg thiamine-HCl, 5.0 mg riboflavin, 5.0 mg nicotinic acid, 5.0 mg sodium pantothenate, 5.0 mg *p*-aminobenzoate, 5.0 mg thioctic acid and trace amounts of cyanocobalamine per 100 mL deionized, distilled water. Wolfe's mineral solution (ATCC, 1992) contained 1.5 g nitrilotriacetic acid, 3.0 g $MgSO_4 \cdot 7H_2O$, 0.5 g $MnSO_4 \cdot H_2O$, 1.0 g NaCl, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $CoCl_2 \cdot 6H_2O$, 0.1 g $CaCl_2$, 0.1 g $ZnSO_4 \cdot 7H_2O$, 0.01 g $CuSO_4 \cdot 5H_2O$, 0.01 g $AlK(SO_4)_2 \cdot 12H_2O$, 0.01 g H_3BO_2, 0.01 g $Na_2MoO_4 \cdot 2H_2O$ and 0.025 g $NiCl_2 \cdot 6H_2O$ per 100 mL deionized, distilled water.

Medium for Fe(III)- and Mn(IV)-reducing microcosms contained 0.1 g $CaCl_2 \cdot H_2O$, 0.1 g KCl, 0.06 g NH_4Cl, 0.6 g $NaH_2PO_4 \cdot H_2O$, 1.0 mL Wolfe's vitamin solution, 1.0 mL Wolfe's mineral solution and 2.5 g $NaHCO_3$ per 1 L deionized, distilled water. After sterilization by autoclaving, a slurry of $Fe(OH)_3$ or MnO_2 was added to these microcosms.

Amorphous $Fe(OH)_3$ was generated by preparing a 0.4 M solution of $FeCl_3$ (6.5 g $FeCl_3$ in 100 mL deionized, distilled water), neutralizing to pH 7 with 10 M NaOH to form a reddish precipitate, then washing with deionized, distilled water until no Cl^- was detectable in the wash solutions, allowing the iron to settle out after each wash (Lovley and Phillips, 1986). To generate MnO_2, two pellets of NaOH were dissolved in 100 mL deionized, distilled water, 1.3 g $KMnO_4$ were added and the pH was brought to near 11. Separately, 2.4 g $MnCl_2 \cdot 4H_2O$ was dissolved in approximately 30 mL deionized, distilled water and added to the $KMnO_4$ solution, forming a blackish precipitate; pH was then adjusted to approximately 9 by adding NaOH pellets. MnO_2 was washed similarly to $Fe(OH)_3$ in order to remove detectable Cl^- (Lovley and Phillips, 1988). For both metal slurries, Cl^- was detected by reacting wash water from the metal slurry with $AgNO_3$. The $AgNO_3$ test involved mixing 1 drop 8.5 g $AgNO_3$ in 100 mL deionized, distilled water with 1 drop supernatant. The presence of Cl^- produced a white precipitate of AgCl. After preparation, metal slurries were suspended in 100 mL deionized, distilled water, bubbled with anaerobic headspace gas and autoclaved in serum bottles before use.

Medium for $SO_4^{=}$ reduction experiments was the Widdel-Pfennig freshwater composition medium (Collins and Widdel, 1986). It contained 960 mL Solution 1, 1 mL Solution 2, 1 mL Solution 3, 30 mL Solution 4, 1 mL Solution 5 and 1 mL Solution 6. Solution 1 consisted of 1.0 g NaCl, 0.4 g $MgCl_2 \cdot 6H_2O$, 0.1 g NH_4Cl, 4.0 g Na_2SO_4, 0.2 g KH_2PO_4, 0.3 g KCl, 0.15 g $CaCl_2 \cdot 2H_2O$ and 10 mL of resazurin solution (0.01 g per 100 mL deionized, distilled water) in 960 mL deionized, distilled water. Solution 2 contained 4.5 mL concentrated HCl, 1.5 g $FeCl_2 \cdot 4H_2O$, 0.06 g H_3BO_3, 0.1 g $MnCl_2 \cdot 4H_2O$, 0.12 g $CoCl_2 \cdot 6H_2O$, 0.07 g $ZnCl_2$, 0.025 g $NiCl_2 \cdot 6H_2O$, 0.015 g $CuCl_2 \cdot 2H_2O$ and 0.025 g $Na_2MoO_4 \cdot 2H_2O$ per 995 mL deionized, distilled water. Solution 3 contained 0.003 g $Na_2SeO_3 \cdot 5H_2O$ and 0.5 g NaOH per 1 L deionized, distilled water. Solutions 1 through 3 were autoclaved. Solution 4 contained 8.4 g $NaHCO_3$ in 100 mL deionized, distilled water and was saturated with CO_2 before being filter sterilized. Solution 5 contained 1.2 g $Na_2S \cdot 9H_2O$ per 10 mL deionized, distilled water. The water was boiled under anaerobic headspace gas before the $Na_2S \cdot 9H_2O$ was added. Solution 6 contained 0.01 g biotin, 0.04 g *p*-aminobenzoate, 0.1 g thiamine-HCl, 0.05 g vitamin B12 and 0.1 g nicotinic acid per 1 L deionized, distilled water and was filter sterilized.

Methanogenic medium was made as described by Fedorak and Hrudey (1984). It contained 1.0 mL Mineral solution I, 0.1 mL Mineral solution II, 1.0 mL resazurin solution, 1.0 mL phosphate solution and 0.57 g $NaHCO_3$ per 100 mL deionized, distilled water. Mineral solution I consisted of 5.0 g NaCl, 1.0 g $CaCl_2 \cdot H_2O$, 1.0 g NH_4Cl, 1.0 g $MgCl_2 \cdot 6H_2O$ per 100 mL 0.01 M HCl. Mineral solution II consisted of 1.0 g $(NH_4)_6Mo_7O_{24} \cdot 2H_2O$, 0.01 g $ZnSO_4 \cdot 7H_2O$, 0.03 g H_3BO_3, 0.15 g $FeCl_2 \cdot 4H_2O$, 1.0 g $CoCl_2 \cdot 6H_2O$, 0.003 g $MnCl_2 \cdot 4H_2O$, 0.003 g $NiCl_2 \cdot 6H_2O$ and 0.01 g $AlK(SO_4)_2 \cdot 12H_2O$ per 100 mL water. Phosphate solution was made up of 5.0 g KH_2PO_4 per 100 mL water and resazurin solution contained 0.01 g resazurin per 100 mL water. Medium was autoclaved before use.

Anaerobic medium based on modified Butlin's medium (S. Ebert, University of Alberta, Edmonton, AB) contained 0.5 g K_2HPO_4, 1.0 g NH_4Cl, 0.003 g $CaCl_2 \cdot 2H_2O$, 1.0 g $MgSO_4 \cdot 7H_2O$, 0.004 g $FeSO_4 \cdot 7H_2O$, 1.0 g NaCl and 10 mL of 0.1 g/L resazurin stock per 990 mL deionized, distilled water. Modifications of this medium were as follows: terminal electron acceptors were provided in the form of $SO_4^{=}$ (2.0 g Na_2SO_4 per 1 L medium), NO_3^- (2.0 g KNO_3 per 1 L medium) or sulfolane (79 μL per 1 L medium; final concentration 100 mg/L), mixed carbon source contained 2.0 g sodium acetate, 0.5 g sodium benzoate, 2.5 g sodium lactate, 1.0 g yeast extract, 4.0 g glucose and 10 mL propionate stock (15% v/v in deionized, distilled water, adjusted to pH 9 with 10 M NaOH) per 1 L medium.

2.4 Chemicals

Chemicals were reagent grade, purchased from Aldrich (Milwaukee, WI), Sigma (St. Louis, MO) or Fisher (Fair Lawn, NJ) unless otherwise indicated. Agar and pre-mixed solid media were obtained from Difco. The solvents used were obtained from the following sources: acetonitrile was HPLC grade from Fisher Chemical; benzene was 99.9% pure HPLC grade from Sigma/Aldrich; chloroform was 99.7% pure, from BDH; dichloromethane was 99.99% pure glass distilled Omnisolv V from EM Science, (Gibbstown, NJ); ethyl acetate was glass distilled, from Caledon Laboratories, Ltd. (Georgetown, ON); methanol was Spectro grade from Caledon; and phenol was laboratory reagent grade from Anachemia (Montreal, PQ). Sulfolane (99%), sulfolene (98%), 3-methylsulfolane (99%), 2,4-dimethylsulfolane (95%), 1.4-butane sultone (99+%), 1-butanesulfonic acid (98% pure sodium salt), benzenesulfonic acid (98% pure sodium salt) and 1-decanesulfonic (98% pure sodium salt) were all obtained from Aldrich. The sulfolane analog 3-hydroxysulfolane was synthesized by P.M. Fedorak (Dept. of Biological Sciences, University of Alberta, Edmonton, AB) from sulfolene. DIPA (98%) was obtained from TCI America (Portland, OR) as a mixture of isomers. Antibiotics (ampicillin, chloramphenicol, kanamycin) were obtained from Sigma. Formaldehyde solution was purchased from Fisher as a 37% w/w solution in water and 10 to 15% methanol.

2.5 Bacterial cultures

Sulfolane-degrading bacterial cultures were isolated from materials obtained from various Sulfinol® plant sites and commercially available soils. Pure bacterial cultures were obtained from both private and commercial sources. These are summarized in Table 2.5. Decreases in the sulfolane concentration in the microcosms and cultures was used as an indicator of biodegradation.

2.6. Treatment of soil cultures and microcosms

Soil cultures and microcosms consisted of soil, wetlands mud, sediment and/or groundwater samples. Samples were placed in sterile containers on site, shipped to the laboratory on ice, and stored at 4°C until used. For inoculation of large-scale microcosms or experiments, soil samples were mixed until uniform, allowing each

Table 2.5. Strains of bacteria used to test the predictive ability of PAT medium (described in Section 2.10.1) for degradation of sulfolane.

bacterial isolate	source of bacterial isolate
Alcaligenes denitrificans	M. Beyer, J. Klein[a]
Bacillus cereus	ATCC[b] 14579
Bacillus polymyxa PKB1	P. Beatty[c]
Bacillus subtilis ssp. niger	E. Bruno[c]
Escherichia coli B15	A. Szenthe[c]
Escherichia coli ESS	A. Wong[c]
Escherichia coli K12 W1485	D. Clark[d]
Escherichia coli K12 NAR11	D. Clark[d]
Micrococcus luteus	ATCC[b] 9341
Nocardia sp.	NCIMB[g] 11399
Proteus vulgaris	E. Bruno[c]
Pseudomonas aeruginosa	A. Szenthe[c]
Pseudomonas alcaligenes	W.R. Finnerty[e]
Pseudomonas fluorescens	A. Szenthe[c]
Pseudomonas putida	M.A. Schell[f]
Pseudomonas putida	NCIMB[g] 8248
Pseudomonas putida	NCIMB[g] 9427
Pseudomonas putida	NCIMB[g] 9816
Pseudomonas putida	NCIMB[g] 12184
Pseudomonas putida	NCIMB[g] 12199
Pseudomonas putida	ATCC[b] 17453
Pseudomonas putida	ATCC[b] 33015
Pseudomonas sp. HL7b	J.M. Foght[c]
Pseudomonas sp.	NCIMB[g] 10643
Rhodococcus sp.	ATCC[b] 29671
Staphylococcus aureus	A. Szenthe[c]
Staphylococcus epidermidis	A. Szenthe[c]
Streptococcus faecalis	ATCC[b] 19433
Streptococcus pyogenes	A. Szenthe[c]

Table 2.5 continued.

bacterial isolate	source of bacterial isolate
Streptomyces clavuligerus	A. Wong[c]
5R	G.S. Sayler, J.M.H King[h]
DFC49	G.S. Sayler, J.M.H King[h]
DFC50	G.S. Sayler, J.M.H King[h]
HL4	J.M. Foght[c]
LMI	G.S. Sayler, J.M.H King[h]
LMII	G.S. Sayler, J.M.H King[h]
RJGII-135	R.J. Grosser, R. Vestal[h]

[a] Weissenfels et al. (1990)

[b] ATCC, American Type Culture Collection, Rockville, MD

[c] Department of Biological Sciences, University of Alberta, Edmonton, AB.

[d] Juhl and Clark (1990)

[e] Finnerty and Robinson (1986)

[f] Yen and Gunsalus (1982)

[g] NCIMB, National Collections of Industrial and Marine Bacteria Limited, Aberdeen, Scotland, U.K.

[h] Foght and Westlake (1991)

separate inoculum to be representative of the site sample as a whole. Materials used for anaerobic experiments were mixed and distributed in an anaerobic chamber with an atmosphere of 5% H_2, 10% CO_2 and 85% N_2. To remove residual H_2, the headspace gas in anaerobic microcosms was replaced after microcosms were inoculated and closed inside the anaerobic chamber. Headspace gas was replaced by applying a vacuum to the sealed serum bottles, then replacing the headspace gas with filter-sterilized O_2-free 30% CO_2, 70% N_2 three times.

2.7 Aerobic microcosms

2.7.1 Experiments to evaluate the sulfolane degradation potential at three contaminated sites

2.7.1.1 Aerobic shake-flask cultures

Cultures containing 200 mL B+NP (low N) medium (Gieg et al., 1998) and aquifer sediment from sites 1, 2 or 3 in a 500-mL Erlenmeyer flask were set up in duplicate, with one sterile control. Cultures with materials from sites 1 and 2 contained 50 g aquifer sediment per flask; cultures from site 3 contained only 35 g aquifer sediment per flask. Cultures were supplemented with a final concentration of 200 mg/L sulfolane. Sterile controls were autoclaved for 30 min on 3 consecutive days, then poisoned with mercuric chloride at a final concentration of 2.5 mg/L (Fedorak and Coy, 1996).

Duplicate cultures and single sterile controls were shaken at 200 rpm at 8°C and at 28°C; 8°C to mimic a typical summer groundwater temperature in Alberta, and 28°C to yield faster degradation rates. Cultures were sampled by removing (1 mL) and frozen at -20°C until analysis.

2.7.2 Population and nutrient requirement screening experiments

Before inoculating the experimental cultures and microcosms, aquifer or wetlands samples were thoroughly mixed, and groundwater samples were mixed in a separate container, then allowed to settle so that solids from the groundwater samples were not included in the experiments. Samples of groundwater were taken for GC analysis to determine the sulfolane and DIPA concentrations present, and for total organic C analysis. These experiments were set up to monitor the biodegradation of both sulfolane and DIPA, therefore both compounds were added to samples when they were not present in groundwater or surface water samples in sufficient quantity. Only the sulfolane biodegradation portion of these experiments will be reported in this thesis.

2.7.2.1 Shake-flask cultures

Shake-flask cultures were comprised of 50 g of sediment and 200 mL of the corresponding groundwater or wetland surface water sample in 500-mL Erlenmeyer flasks. East and west plume groundwaters contained sufficient sulfolane and DIPA for the experiment, however the uncontaminated and wetland water samples did not contain detectable amounts of sulfolane or DIPA. These cultures were spiked with a final concentration of approximately 120 mg/L of each compound.

For each sampling site, four cultures were monitored: one sterile control, one unsupplemented culture and two cultures to which supplemental N and P sources were added to stimulate biodegradation. The stock solution of N and P supplement contained 1.2 g NH_4Cl, 2.0 g KNO_3, 32 g KH_2PO_4 and 48 g K_2HPO_4 per L of water. For one set of N- and P- supplemented cultures, initially 1 mL of this stock solution was added, providing 3.1 mg N/L and 79 mg P/L. After 14 d of incubation, a further 9 mL of the stock solution were added, increasing the total supplemented concentrations to 31 mg N/L and 790 mg P/L. The total supplemented concentrations of 31 mg N/L and 790 mg P/L were added at time zero to the second N- and P- supplemented cultures. This provided the same time zero N and P concentrations as used in the population screening experiments described previously (Sections 2.1.1, 2.7.1). Cultures were incubated shaking at 200 rpm at 8°C and monitored for 161 d. Samples (1 mL) were taken; pH was measured immediately and samples were then stored frozen at -20°C until analyzed.

Sterile control cultures were autoclaved for 30 min and poisoned with a final concentration of 250 mg/L formaldehyde (125 µL of 37% w/w formaldehyde solution). A second 125-µL portion of formaldehyde was added at day 9, giving a final formaldehyde concentration of 500 mg/L.

2.7.2.2 N- and P-supplemented shake-flask cultures

The initial shake-flask studies demonstrated that N and P supplementation was particularly important for sulfolane biodegradation by microorganisms in aquifer sediment materials. The specific effects of N or P supplementation on biodegradation of sulfolane were therefore examined, using east plume and west plume aquifer materials.

A C:N:P ratio of 100:5:1, by weight (Alef and Nannipieri 1995), based on the C contributed by sulfolane and DIPA in the west plume groundwater, was used as the basis for the N and P concentrations that were tested. The mean sulfolane and DIPA concentrations in the west plume groundwater samples were 680 mg/L and 70 mg/L, respectively, giving organic carbon concentrations of 272 mg/L and 38 mg/L (a total of

310 mg/L) in the groundwater. The N contributed by DIPA was not included in the calculations. The initial set-up was based on a 3 x 3 experimental matrix with added nutrient concentrations (for the west plume) of $0N_r$, $1N_r$ and $2N_r$ by $0P_r$, $1P_r$ and 2Pr, where $1N_r$ and $1P_r$ are the amount of N or P, respectively, calculated to give a C:N:P ratio of 100:5:1. One sterile control flask with no added N or P and a positive control flask with $2N_r$ and 255 P_r were also incubated. The positive control N and P concentrations were the same as those in the previous experiments (Sections 2.7.1 and 2.7.2.2).

The total organic C contributed to the east plume groundwater by sulfolane and DIPA was 197 and 188 mg/L, respectively. Due to this difference in carbon concentration from the west plume groundwater, the N and P supplementation resulted in a matrix of $0N_r$, $0.8N_r$ and $1.6N_r$ by $0P_r$, $0.8P_r$ and $1.6P_r$. Three extra cultures supplemented with $1.6N_r$ and $2.4P_r$, $3.2P_r$ or 4.0 P_r, a sterile control culture with no added N or P, and a positive control culture containing $1.6N_r$ and $205P_r$ were also incubated.

2.7.2.3 2.5-L microcosms

Microcosms modeled after those described by Holm et al. (1992) and Nielsen and Christensen (1994) were prepared by transferring 1 kg of aquifer sediment and 1.3 L of the corresponding groundwater or 1 kg of wetland material and 1.3 L of the corresponding surface water to a 2.5-L bottle. Groundwater and surface water were aerated to approximately 10 mg/L dissolved O_2 by bubbling air through the water sample, then measured into the microcosms. Water samples added to control microcosms were not aerated. The groundwater samples corresponding to the east plume and west plume were not modified, however water from the other three were spiked a final concentration of approximately 120 mg/L each of sulfolane and DIPA in the microcosms.

The east plume and west plume microcosms and both wetland microcosms had a corresponding sterile control. Insufficient material was provided to allow a sterile control for the uncontaminated aquifer sediment microcosm. For the sterile controls, all the soil and one half the volume of groundwater were autoclaved in the 2.5-L microcosm bottle, while the other half the groundwater was autoclaved separately, then added to the microcosm bottle. Sterile controls were autoclaved for 30 min, then poisoned with 810 µL of 37% formaldehyde. A second addition of 810 µL of the formaldehyde solution into each sterile microcosm on day 8 increased the overall concentration of formaldehyde to 500 mg/L. The second dose of formaldehyde solution was added because some sulfolane concentrations in the sterile controls appeared to decrease over time. All samples were incubated at 8°C, shaking at 40 rpm. Samples (1 mL) were taken from the liquid layer of the microcosms and the pH was measured immediately, then samples were stored at -20°C until analysis. The dissolved O_2 concentration in the viable microcosms was measured using a dissolved O_2 probe (Section 2.2.8).

2.7.2.4 Respirometer microcosms

The O_2 demand during sulfolane and DIPA degradation by microcosms containing aquifer sediments and groundwaters from the east plume or uncontaminated region was measured using a Comput-Ox 00-244 Respirometer obtained from J.P. Salanitro (Shell Development Company, Houston, TX). The respirometer was supplied and maintained by N-Con Systems Co. Inc (Crawford, GA). Microcosm bottles were made for the experiment by adding a glass sampling port at a 45° angle to the shoulder of a 500-mL Pyrex bottle (Corning, Horseheads, NY). Each sampling port was sealed with a rubber septum (Figure 2.2). Each bottle was connected to a pressure transducer and valve assembly attached to a cylinder of O_2. As O_2 was consumed, the pressure in the headspace of the microcosm decreased (Salanitro et al., 1997), triggering influx of O_2 to maintain the atmospheric pressure in the vessel. A KOH pellet trap was used to remove CO_2 as it formed, ensuring that changes in O_2 would be the only factor contributing to atmospheric pressure change in the microcosms.

Four microcosms from the east plume, two from the uncontaminated region and two sterile control microcosms containing material from the uncontaminated region were prepared. All microcosms containing uncontaminated samples were spiked with a final concentration of approximately 200 mg/L each of sulfolane and DIPA. Microcosms consisted of 50 g of soil and 400 mL of the corresponding groundwater. For the sterile controls, the soil was autoclaved for 35 min and separately autoclaved groundwater was added, then the controls were poisoned by adding a final concentration of 2.5 mg/L mercuric chloride. All samples were allowed to equilibrate at 11°C and then sparged with 5% O_2, 95% N_2 while mixing, before they were sealed. This O_2 concentration was predicted to be best for maintaining a dissolved O_2 concentration of approximately 2.5 mg/L (2.35 to 2.75 mg/L) during the experiment; the concentration of O_2 in the initial incoming gas affected the dissolved O_2 in the microcosms (Salanitro et al., 1997). The microcosms were incubated at 12°C, stirring constantly. Samples (1 mL) were removed through the sampling port in each microcosm bottle during the incubation period, using a

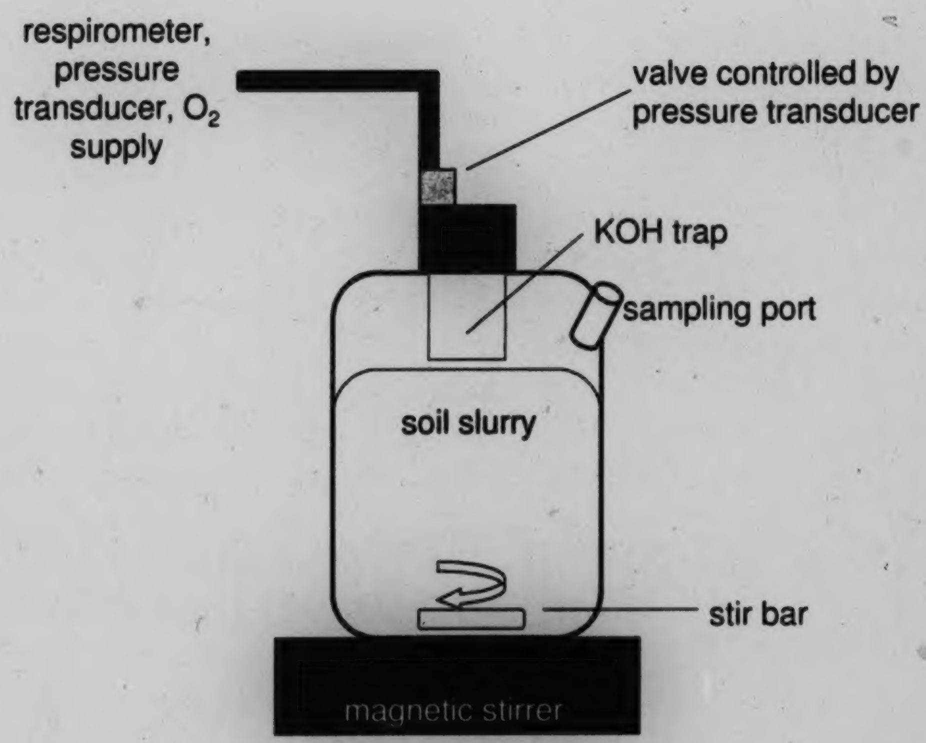

respirometer, pressure transducer, O₂ supply
valve controlled by pressure transducer
KOH trap
sampling port
soil slurry
stir bar
magnetic stirrer

Figure 2.2. Diagram of a microcosm bottle used in the respirometer experiment.

25 gauge, 8 cm needle and a 5-mL syringe (Beckton Dickinson, Rutherford, NJ), and after the pH was measured the samples were frozen at -20°C until analysis.

2.7.3 Air-sparged microcosm experiments

Laboratory microcosms used to simulate an air-sparged field situation were designed based on the method of Mueller et al. (1991), as modified by Fedorak and Coy (1996). Corresponding shake-flask cultures were used to test whether the shake-flask results could be used to predict microbial activity in a simulated field situation. Two types of materials were used for this experiment: a commercially available topsoil and a sulfolane-contaminated sediment taken from site 2 near a Sulfinol® plant building.

2.7.3.1 Shake-flask cultures

Shake-flask cultures containing topsoil or sediment were used to predict which nutrients would be required to stimulate sulfolane biodegradation in the air-sparged microcosms. Nutrient supplementation was calculated based on the C:N:P ratio of 100:5:1, such that $1N_r$ and $1P_r$ were the amount of N and P required to provide this ratio with respect to the C source described. Shake-flasks inoculated with contaminated sediment were tested under the following conditions: $1N_r$, $1P_r$ and $1N_r + 1P_r$ for sulfolane present in the culture, $1N_r$, $1P_r$ and $1N_r + 1P_r$ for total C present in the culture, no N or P, and a sterile control. Samples inoculated with aquifer sediment did not require the addition of sulfolane; when 50 g of soil was shaken for 30 min in 200 mL of water the resulting concentration was 160 to 170 mg/L sulfolane. The N_r calculated for sulfolane alone in the contaminated sediment sample was 15 µL 50 g/L N solution (143 g/L NH_4NO_3); the P_r calculated for sulfolane was 3.6 µL 40.6 g/L P (100 g/L K_2HPO_4 and 100 g/L KH_2PO_4). The N_r for total organic C in each contaminated sediment culture was supplied by adding 1.12 mL of the 50 g/L sterile N stock solution, and the P_r by adding 275 µL of the 40.6 g/L sterile P stock solution. The topsoil cultures were incubated with the same proportions of N_r and P_r to sulfolane-C or to total organic C as the contaminated sediment cultures. These cultures were also spiked with a final sulfolane concentration of 200 mg/L. The N stock used to supplement the topsoil cultures contained 6.48 g NH_4Cl and 10.8 g KNO_3 per 1 L deionized, distilled water (3.2 g/L N) and the P stock contained 12.4 g KH_2PO_4 and 18.6 g K_2HPO_4 per 1 L deionized, distilled water (0.62 g/L P). For the cultures where N_r and P_r were calculated based on C from sulfolane, 253 µL of N, P, or N and P solution was added. For topsoil cultures where N_r and P_r was calculated based on total organic C present, 1 N_r was added in the form of 11.5 mL 50 g/L N stock and $1P_r$ was represented by 2.8 mL of 40.6 g/L P stock.

Shake-flask cultures consisted of 50 g of sediment or topsoil in 200 mL sterile deionized, distilled water. The soil or sediment present in each culture was predicted to provide sufficient buffering to allow cell survival in the water. The sterile control cultures for both soil types were autoclaved for 30 min after the addition of the soil inoculum, then poisoned with 250 μL of 37% v/v formaldehyde. Cultures were incubated shaking at 200 rpm, at room temperature (22°C). Samples of 1 mL were taken and frozen at -20°C until analysis.

2.7.3.2 Air-sparged microcosms

For air-sparged microcosms, a uniform sample of soil or contaminated sediment was prepared, separated into four portions, then modified with the appropriate nutrient solutions. Four microcosms were set up for each soil type; an unsupplemented microcosm, one supplemented with N, one with P and one with N and P. The air-sparged microcosm setup is shown in Figure 2.3. Microcosms consisted of a 3-L Pyrex Buchner funnel (Kimble), lined with sterile glass wool (approximately 1 cm thick when packed), which was covered with a sterile section cut from an aluminum foil pie plate, perforated with approximately 20 holes, each 2-3 mm in diameter. Funnels were sterilized by washing them with ethanol, then exposing them to UV light overnight, before the glass wool and foil were put in place. After inoculation with soil, each funnel was covered with a small Pyrex dish to reduce moisture loss from the system. The bottom of each funnel was attached to a piece of rubber tubing and a sterile filter. The tubing from each of the four funnels was connected together with y-joints, then attached to the house air system (40 psi), via a a 2-L flask containing water, which was used to moisturize the air (Figure 2.3). During the incubation period the funnels stood in two custom-designed wooden support boxes, each built to hold two funnels.

Air-sparged microcosms were sampled over time by thoroughly mixing the contents of each microcosm with a sterile spatula, then removing 7 or 8 g soil to a sterile, disposable centrifuge tube. From this sample, 1 g was used for plate counts, 3 g for three replicate sulfolane extractions and1 g for determining the soil dry weight:wet weight ratio.

Total cultureable heterotrophs and sulfolane-degrading bacteria were estimated by diluting 1 g of soil in 10 mL of sterile deionized, distilled water, then plating serial dilutions on PAT medium (described in Section 2.10.1). Plates were incubated at room temperature. For the topsoil microcosms, single replicates were used. For the contaminated sediment microcosms, each sample was plated in triplicate. Total colony

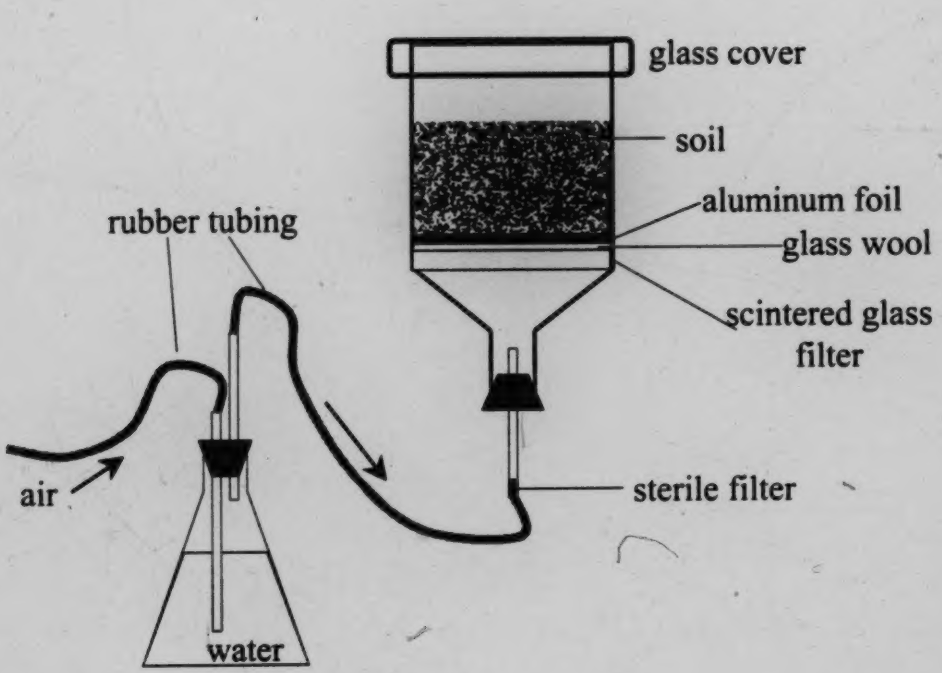


Figure 2.3. Diagram of an air-sparged microcosm (similar to the landfarming chamber microcosm described by Mueller et al., 1991) used to simulate an aerated field situation in the laboratory.

counts were done after the PAT plates had incubated for 14 d, while sulfolane-degrading colonies were not counted until 21 to 28 d after plating.

The amount of sulfolane present in each soil sample was measured in triplicate at each sampling time. For each extraction, 1 g soil was measured into a sterile, 15-mL disposable centrifuge tube, vortexed with 5 mL deionized, distilled water until fully resuspended, then centrifuged in a clinical centrifuge at maximum speed (approximately 2800 rpm or 890 x g) for 10 min and the supernatant collected. This process was repeated once; the two 5-mL supernatant samples were combined, mixed, and a 1-mL sample of each was frozen at -20°C until analyzed. These samples were also used to measure water-extractable total organic C.

The dry weight:wet weight ratio was calculated each time the soil columns were sampled. Approximately 1-g samples of soil were weighed, wrapped in aluminum foil and heated at 105°C for 7 d, then weighed a second time. The soil dry weight was divided by the wet weight to determine the dry:wet weight ratio. All sulfolane concentrations and cell counts were multiplied by this ratio and reported per g soil dry weight.

Microcosms with contaminated sediment contained only 0.5 kg of sediment due to the small amount of sample available; the remainder of the sediment was stored at 4°C until used to inoculate shake-flask experiments. Four soil microcosms were incubated: one unamended, one amended with N, one amended with P and one amended with N and P. The sediment was contaminated with sulfolane at a concentration of approximately 600 mg/kg soil (determined by extractions of soil previous to inoculation). A C:N:P ratio of 100:5:1, using the C contributed by sulfolane, was used to determine the amount of N or P to add to each sample. The N and P were added in 10 mL of sterile deionized, distilled water so that they could be evenly distributed throughout each soil sample by mixing.

Microcosms amended with N were given 60 µL of a 50 g/L N stock; P was given to the appropriate microcosms as 15-µL of a 40.6 g/L P stock (Section 2.7.3.1). The unamended sample was mixed with 10 mL of deionized, distilled water with no added N or P.

The total C present in the soil was 2.23% (Table 2.3), therefore 11.2 mL of the 50 g/L N stock and 2.8 mL of the 40.6 g/L P stock were added to the appropriate samples after 21 d of incubation. Samples were transferred from the soil columns into sterile

Pyrex dishes for mixing. A total of 20 mL sterile deionized, distilled water or water mixed with N and/or P solution was added to each sample at this time.

Contaminated sediment from the Sulfinol® plant building contained strong-smelling volatile compounds, therefore it was necessary to incubate microcosms containing these materials in a fume hood. Due to the high level of air circulation in the fume hood, moisture in the samples was lost rapidly, therefore sterile deionized, distilled water was added to the columns weekly or biweekly during the incubation. Mueller et al. (1991) also found it necessary to add water to air-sparged microcosms during incubation.

To prepare the air-sparged microcosms inoculated with topsoil, 7 kg of topsoil was thoroughly mixed, then 1.5 kg of topsoil was transferred into each of four sterile Pyrex dishes and the remainder of the soil was stored at 4°C until used to inoculate the shake-flask experiments. Microcosms were amended by adding sulfolane and the appropriate nutrient solutions to each sample and mixing until they were completely distributed. Each of the four topsoil samples was amended with a final sulfolane concentration of 200 mg/kg soil. The amount of N and P added to each microcosm was calculated for the sulfolane added to each microcosm, based on a C:N:P ratio of 100:5:1. The topsoil samples amended with N or N and P were given 1.9 mL of a sterile stock solution containing 6.48 g NH_4Cl and 10.8 g KNO_3 per 1 L deionized, distilled water. The samples amended with P or N and P were each given 1.9 mL of a sterile stock solution containing 12.4 g KH_2PO_4 and 18.6 g K_2HPO_4 per 1 L deionized, distilled water. Topsoil microcosms were reamended; initially the samples amended with N or N and P were reamended with the same concentrations of sulfolane and N or N and P that were initially added. All four samples were later reamended with 15 mL of 100 g/L sulfolane. The appropriate samples were also reamended with N or P; 600 μL N stock solution (143 g NH_4NO_3 per 1 L deionized, distilled water) or 148 μL P stock (100 g K_2HPO_4 and 100 g KH_2PO_4 per 1 L deionized, distilled water). Soil columns modified with N or N and P were modified again with the higher concentrations of sulfolane, N and/or P.

2.8 Determination of kinetic constants

In order to better compare sulfolane biodegradation data obtained under various conditions, biodegradation rates were calculated, using the method outlined by Metcalf and Eddy (1979); each set of substrate removal data was plotted in two ways to determine whether it better fit zero-order or first-order kinetics (Appendix B, Figure B1).

A more complex analysis of biodegradation kinetics was not important for the level of comparison carried out in this thesis project. The method of Metcalf and Eddy (1979) is described in the following paragraph.

Zero-order kinetics are described by a plot of substrate concentration vs time that is linear. The slope of this line is the rate constant, k_0, in mg/L/h. The initial substrate concentration divided by $2k_0$ will give the half-life of the compound in the system, in h. First-order kinetics exist when the plot of the natural logarithm of substrate concentration vs time is linear. The half-life of the compound in a system described by first-order kinetics is equal to ln [2] divided by the slope of the line, which is the rate constant k_1 (in h^{-1}). To calculate whether sulfolane biodegradation in a culture or microcosm was better described by zero-order or first-order kinetics, the portion of the data describing a decrease in sulfolane was plotted against time in mg/L and again in ln [mg/L]. The fit of the line with the data points was evaluated by the R^2 for each plot, where an R^2 of 1 was a perfect fit of the line to the data points; $R^2 < 1$ indicated some deviation of the data points from the best fit line. For each data set, the graph with the higher R^2 value was determined to better describe the kinetics of sulfolane removal from the culture. An example of kinetic rate plots is shown in Figure B1 (Appendix B).

2.9 Anaerobic microcosms

In all the anaerobic microcosms, a decrease in sulfolane concentration was considered to be an indicator of sulfolane biodegradation. Changes in terminal electron acceptors in the microcosms were also monitored to ascertain whether they were utilized.

2.9.1 Anaerobic experiments to evaluate the sulfolane degradation potential at three contaminated sites

2.9.1.1 Sulfolane-containing microcosms

Anaerobic microcosms were set up in 158-mL serum bottles. Sterile anaerobic media were prepared as described above (Section 2.3.1). For NO_3^--reducing, $SO_4^{=}$-reducing and methanogenic microcosms, 75 mL of medium was placed in each serum bottle. For Fe(III)-reducing and Mn(IV)-reducing microcosms, 65 mL of medium was placed in each serum bottle; 10 mL of the appropriate oxidized metal slurry was added at the time of inoculation. Sterile media were placed in an anaerobic chamber with an atmosphere of 5% H_2, 10% CO_2 and 85% N_2 for inoculation. In the anaerobic chamber, aquifer sediments were aseptically transferred into the serum bottles. Serum bottles were then re-sealed, removed from the anaerobic chamber, and the headspace gas was changed to O_2-free 30% CO_2, 70% N_2 (Section 2.3.1). Microcosms were amended with sulfolane by injecting 0.5 mL of a sterile, anaerobically prepared 30 g/L sulfolane stock solution. Sterile controls were autoclaved for 30 min on 3 consecutive days, then poisoned with chloroform at a final concentration of 0.5% v/v (Albrechtsen et al., 1995). Duplicate microcosms and a single sterile control were incubated statically at 8°C and at 28°C. Samples were taken from each microcosm by shaking thoroughly to resuspend the solids in the medium, injecting 1 mL O_2-free 30% CO_2, 70% N_2, then removing 1 mL aquifer sediment slurry. Samples were stored frozen at -20°C until analysis.

2.9.1.2 Acetate-containing microcosms

Acetate-containing microcosms were set up in a similar manner to the sulfolane-containing anaerobic microcosms (Section 2.9.1.1); however microcosms were each injected with 0.5 mL of a 0.75 M sterile stock solution of sodium acetate. Sterile controls were made in the same manner as sulfolane-containing sterile controls.

Two acetate-degrading positive control microcosms were incubated for each type of terminal electron acceptor: one inoculated with a culture known to utilize acetate in the presence of that terminal electron acceptor, and one inoculated with the same active culture and with sediment from site 1, 2 or 3, so that any potential inhibition of the active population by compounds present in the sediment could be observed. The microbial inocula for the acetate-utilizing positive controls were as follows: *Pseudomonas* sp. W1 (Kropp et al., 1994) for NO_3^--reducing conditions, *Geobacter metallireducens* (Gieg, 1996) for Mn(IV)- and Fe(III)-reducing conditions, *Desulfobacter hydrogenophilus* for $SO_4^=$-reducing conditions and fresh anaerobic sewage sludge (Goldbar Wastewater Treatment Plant, Edmonton, AB) for methanogenic conditions. Duplicate microcosms, each of the positive control microcosms, and a single sterile control were incubated statically in the dark at 28°C and at 37°C, whichever temperature was deemed best for the bacterial inoculum. Samples were taken from each microcosm as described in Section 2.9.1.1 and stored frozen at -20°C until analysis.

2.9.1.3 Sulfolane-containing anaerobic sewage sludge microcosms

Fresh, anaerobic sewage sludge from the Goldbar Wastewater Treatment Plant was added to sterile 158-mL serum bottles under a headspace gas of 30% CO_2 70% N_2. A total of 18 bottles were inoculated with 75 mL of sewage sludge each. The bottles were sealed and modified with either sulfolane or acetate as a C source. Each sulfolane or acetate concentration was tested in triplicate. Microcosms modified with sulfolane were injected with 0.125, 0.25, or 0.5 mL of 30 g/L sterile sulfolane prepared under anaerobic conditions. Microcosms modified with acetate were injected with 0.125, 0.25 or 0.5 mL of a sterile stock solution of 0.75 M sodium acetate, prepared under anaerobic conditions. Methane, acetate and/or sulfolane were monitored by GC.

2.9.2 Anaerobic microcosms used to test the ability of sulfolane to act as a terminal electron acceptor

2.9.2.1 Sediment-containing microcosms

Two sets of sediment-containing microcosms were used to determine whether sulfolane is able to act as a terminal electron acceptor. Both were inoculated with sediment from the Sulfinol® plant building samples (described in Tables 2.3 and 2.4 and Section 2.1.4). The first series of microcosms contained Sulfinol® building #1 sediment. A total of 10 samples, each containing 25 g soil and 0, 5, 10, 15 or 20 mL sterile deionized, distilled water, incubated at room temperature in sterile 38-mL serum bottles. One sample series was spiked with 60 µL of 100 g/L sterile sulfolane stock, to give a final sulfolane concentration of approximately 250 mg/kg sediment; the second sample series was not supplemented with sulfolane. Microcosms were inoculated in an anaerobic chamber with an atmosphere of 5 % H_2, 10 % CO_2 and 85 % N_2. These microcosms were designed for sampling of headspace gas only; little or no water was added to the sediment, therefore no sediment or sediment and water slurries were removed during the incubation period.

The second series of contaminated sediment-containing microcosms tested the ability of microorganisms in sediments taken near Sulfinol® building #1 and Sulfinol® building #2 to reduce sulfolane. For each microcosm, a sterile 38-mL serum bottle was inoculated with 25 g soil and 10 mL sterile deionized, distilled water in the anaerobic chamber described above. Sediment taken from Sulfinol® building #1 contained approximately 94 mg sulfolane/kg soil, while Sulfinol® building #2 sediment contained approximately 250 mg sulfolane/kg soil. The Sulfinol® building #1 sediment microcosms were amended with: 125 µL 100 g/L sterile sulfolane stock (triplicate microcosms incubated), 125 µL 20 % w/v sterile sodium acetate stock and 125 µL 100 g/L sterile sulfolane stock (triplicate microcosms incubated), and one each of a sterile control modified with either sulfolane or sodium acetate and sulfolane. The Sulfinol® building #2 sediment microcosms were amended with: no added nutrients (triplicate microcosms incubated), 125 µL 20% sodium acetate (triplicate microcosms incubated), 125 µL 100 g/L sulfolane (one microcosm incubated), and one each of a sterile control with either no added nutrients or with 125 µL 20% sodium acetate. Sterile control microcosms were made by autoclaving for 30 min, then poisoning with 125 µL 37% v/v formaldehyde. Microcosms were incubated at room temperature. Samples (0.5 mL) were taken using a 1-mL syringe equipped with a 16 g needle. Headspace gas was replaced with 30% CO_2, 70% N_2 when samples were removed. Samples were stored frozen at -20°C until analyzed.

2.9.2.2 Pure or mixed culture microcosms

These microcosms were set up to determine whether a known sulfolane-degrading pure culture (WPW-S28, described in Section 2.11.2.1), an *Escherichia coli* K12 culture predicted to reduce tetrahydrothiophene sulfoxide (Juhl and Clark, 1990), a pure $SO_4^=$-reducing culture, or a mixed $SO_4^=$-reducing culture were able to degrade or reduce sulfolane under anaerobic conditions. For each of the four cultures, five different nutrient and terminal electron acceptor combinations were tested, using two replicate microcosms and one sterile control for each condition. The test conditions were as follows: sulfolane as a C source with NO_3^- or $SO_4^=$ as a terminal electron acceptor, or a mixed C source with sulfolane, NO_3^- or $SO_4^=$ as a terminal electron acceptor. The medium was a modification of Butlin's medium (described in Section 2.3.2) that contained no source of C or terminal electron acceptor. Sulfolane was added to the appropriate microcosms at a final concentration of 100 mg/L. Sulfate was added to microcosms as 2.0 g Na_2SO_4 per 1 L medium, NO_3^- as 2.0 g KNO_3 per 1 L medium. Microcosms were inoculated using a syringe, in order to avoid introducing O_2.

The *E. coli* culture used in this experiment was *Escherichia coli* K12, obtained from T. Hantos (Dept. of Biological Sciences, University of Alberta, Edmonton, AB). This culture was grown in four cultures of 200-mL LB medium. Cells were incubated overnight, shaking at 200 rpm, 28°C, then harvested by centrifugation at 10 400 x g for 20 min. Cell pellets were resuspended in 50 mL 50 mM sterile KH_2PO_4, centrifuged a second time, then resuspended once more in 200 mL 50 mM sterile KH_2PO_4. Each microcosm was inoculated with 5 mL of the resulting cell suspension.

WPW-S28 was grown for 7 d in B+N8P with 500 mg/L sulfolane. Twenty 200-mL cultures were incubated at 28°C, shaking at 200 rpm. Once sulfolane removal was complete, the cells were centrifuged at 10 400 x g for 20 min. When all the cells had been collected, each cell pellet was resuspended in 50 mL 50 mM sterile KH_2PO_4, and centrifuged at 10 400 x g for 20 min. The resulting cell pellets were combined in a total of

150 mL 50 mM KH_2PO_4. Each microcosm was inoculated with 5 mL of the resulting cell suspension.

The mixed and pure $SO_4^=$-reducing cultures were provided by S. Ebert (Dept. of Biological Sciences, University of Alberta, Edmonton, AB). The mixed culture was a $SO_4^=$-reducing enrichment from a methanogenic pond at the Suncor oil sands project, taken from a depth of 10 m. The pure culture was *Desulfovibrio vulgaris* var. *oxamicus*. In both cases, the cultures were grown in 10 mL modified Butlin's medium, and 0.5 mL was used to inoculate each of the microcosms used in the experiment. Cultures were provided in 18 mm glass test tubes, therefore to avoid exposing them to air, the microcosms containing $SO_4^=$-reducing bacteria were inoculated in the anaerobic chamber. Cultures were monitored by taking samples (0.5 mL); headspace gas was replaced with 30% CO_2, 70% N_2 when samples were removed. Samples were stored frozen at -20°C until analyzed.

2.10 Medium to detect sulfolane-degrading organisms

2.10.1 PAT medium for detecting sulfolane-degrading organisms

A differential medium was designed to detect sulfolane-degrading microorganisms; this was called pH agar for tetrahydrothiophene-1,1-dioxide (PAT). PAT medium consisted of 2.3 g Bacto plate count agar (PCA, Difco Laboratories, Detroit, MI), 17 g Bacto Agar (Difco), 0.1 g bromthymol blue (Sigma Chemical Co.) and 1 g sulfolane per 1 L of deionized, distilled water.

2.10.2 Bacterial strains used to test PAT medium

Bacterial strains were tested for their ability to grow on PAT medium. The isolates and their sources are listed in Table 2.5. Bacterial cultures used to test PAT medium were maintained on PCA plates or in LB or 2 x YT liquid medium (Sambrook et al., 1989). 'Positive' sulfolane-degrading colonies were indicated by the development of a yellow halo (indicating acid production) around each colony. Cultures were incubated at 28°C or at room temperature; laboratory strains were incubated at the higher temperature and environmental isolates at the lower temperature. Strains which appeared positive for sulfolane degradation were tested in liquid medium to confirm this ability. Overnight cultures of the test bacterium were grown in 10 mL LB in test tubes rotating at 50 rpm at 28°C or room temperature. This culture was used to inoculate 200 mL of B+N8P containing 200 mg/L sulfolane. Cultures were incubated shaking at 200 rpm in the dark for 30 d; 1-mL samples were removed over this time period for measurement of OD_{600} and sulfolane concentration. Samples were frozen at -20°C until sulfolane analysis was performed.

2.10.3 Method for testing bacteria isolated on PAT medium

Sulfolane liquid medium consisted of $SO_4^=$-free medium supplemented with 500 mg/L sulfolane and 0.05 mg/L bromthymol blue pH indicator. Cultures were incubated in 10 mL medium in 18 mm x 150 mm test tubes rotating at 50 rpm, at 28°C. For each culture, a 0.2-mL sample was taken at time zero for analysis of sulfolane concentration. Samples (0.2 mL) were analyzed a second time when the medium became acidic; at this time the cultures were neutralized with sterile 0.2 N NaOH. Samples were analyzed once more when the cultures became acidic a second time. Cultures in which no color change was observed were sampled and analyzed at the end of the experiment. At the time of inoculation, each colony tested was inoculated in the liquid medium tube, then streaked on LB agar to determine whether that colony contained viable cells and PAT medium to test whether they appeared to degrade sulfolane.

2.11 Mixed and pure bacterial enrichment cultures

Pure sulfolane-degrading organisms were isolated by enrichment from sulfolane-contaminated sediments. Culture names were based on the site from which they were originally maintained, the culture purity and the temperature at which they were enriched. Cultures were named using the following formula: ABC-SD, where A represented the first letter of the sampling site name, B was either M for a mixed culture or P for a pure culture, C represented the isolate number from that sample, or the region of the site from which the sample was taken, S indicated that the culture was isolated or enriched on sulfolane and D represented the temperature at which the culture was incubated. The pure and mixed cultures discussed in this thesis are summarized in Table 2.6.

2.11.1 Aerobic mixed cultures

2.11.1.1 Initial enrichments

Enrichment cultures were made from sulfolane-containing sediments, using sediment inocula in mineral medium. After sulfolane had been removed from the initial culture, it was reamended with a final concentration of 200 mg/L sulfolane. When sulfolane was removed a second time, 20 mL of the sediment slurry was transferred to a fresh flask of sterile medium, containing 200 mg/L sulfolane. Further enrichments were made by continuing to inoculate fresh medium with 10% (v/v) of the previous enrichment, after sulfolane had been completely removed from the previous enrichment culture. Enrichment cultures were all maintained in B+N8P medium modified with 500 mg/L sulfolane, and were generally transferred every 4 wk.

2.11.1.2 Growth of aerobic mixed cultures

Aerobic mixed cultures, using sulfolane as a sole source of C and energy in B+N8P medium, were monitored over time using OD_{600} to measure cell growth and GC to follow the loss of sulfolane. In order to determine whether sulfolane could also act as a sole source of S, or to prepare cultures for C and S balance experiments (Section 2.12), $SO_4^{=}$-free medium was also used for mixed culture growth. The pH of these cultures was monitored; when the pH dropped they were neutralized by adding sterile 0.2 M NaOH.

2.11.1.3 Effect of sulfolane analogs on aerobic mixed cultures

The effect of five sulfolane analogs on the ability of mixed sulfolane-degrading cultures to degrade sulfolane, and the ability of these cultures to degrade the sulfolane analogs, was tested. The compounds tested were 3-methylsulfolane, 2,4-dimethylsulfolane, 3-hydroxysulfolane, butane sultone, and sulfolene (Figure 2.4). These compounds were dissolved in deionized, distilled water and filter sterilized before being added to B+N8P medium at a final concentration of 200 mg/L. The medium was then inoculated with a mixed bacterial culture (10% v/v) and incubated shaking at 200 rpm, 28°C. Compounds were either tested alone or in the presence of 200 mg/L sulfolane. Culture growth was monitored by OD_{600}; sulfolane and sulfolane analogs were analyzed by GC. Cultures which did not appear to grow or to remove any compounds from the medium were plated on LB agar in order to ensure that viable cells were present.

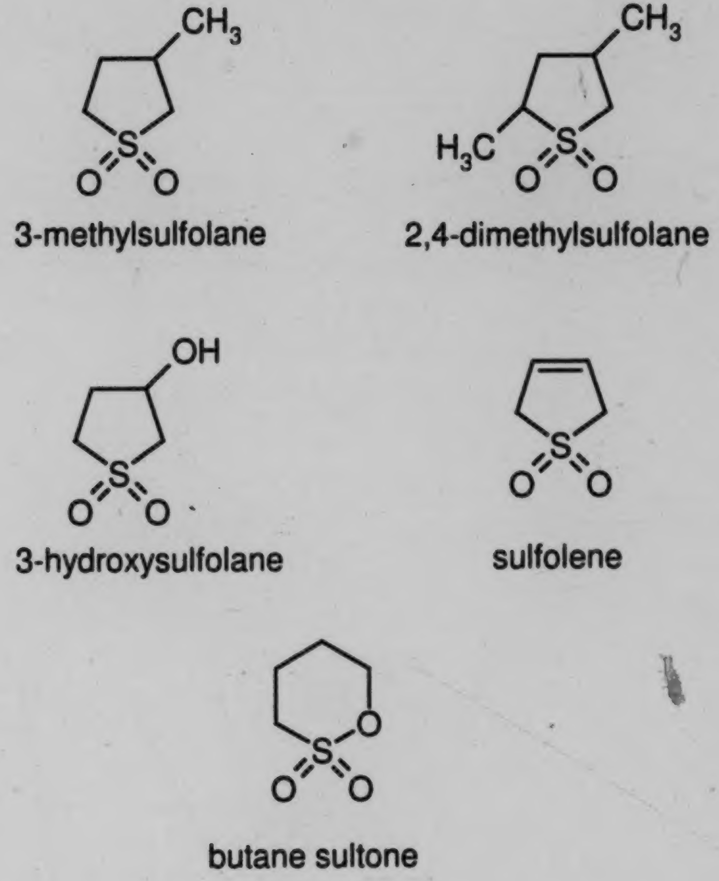
2.11.2 Aerobic pure cultures

2.11.2.1 Isolation of pure, aerobic sulfolane-degrading cultures

PAT medium (described in Section 2.10.1) was used to detect sulfolane-degrading bacteria. Bacterial isolates that appeared to degrade sulfolane on PAT medium were inoculated into test tubes containing 10 mL B+N8P with 500 mg/L sulfolane, and streaked onto a fresh plate of PAT medium and an LB agar plate to ensure purity. Sulfolane-degrading, pure cultures each were used to inoculate 200 mL of B+N8P medium with 500 mg/L sulfolane. Maintenance cultures were transferred (a 10% v/v inoculum in fresh medium) approximately every 4 wk. Cultures were also grown in $SO_4^{=}$-free medium (Fedorak and Coy, 1996).

Table 2.6. Designations for the mixed and pure bacterial cultures enriched and/or isolated from sites 1 (H) or 2 (W). Cultures were named on the following basis: the sample site is indicated with the first letter, the second letter is M for mixed cultures, P for pure cultures, the remainder of the letters and numbers before the dash are individual culture identifiers; after the dash S indicated a sulfolane-degrading culture enriched on sulfolane as a sole source of C and energy and the number indicates the temperature of incubation in °C.

mixed cultures (site 1)	pure cultures (site 1)	pure cultures (site 2)
HMR-S28	HPRK2-S28	WP1-S28
HM3-S28	HP1-S28	WPES-S28
HM5-S28	HP3-S8	WPS-S28
HM8-S8	HP5-S8	WPU-S28
	HP8-S28	WPW-S28
	HP81-S8	WPWS-S28



3-methylsulfolane

2,4-dimethylsulfolane

3-hydroxysulfolane

sulfolene

butane sultone

Figure 2.4. Chemical structures of some sulfolane analogs.

2.11.2.2 Growth of aerobic pure cultures on sulfolane

Aerobic pure cultures were grown in B+N8P, B+NP, or $SO_4^=$-free medium. The change in OD_{600} during sulfolane biodegradation for pure sulfolane-degrading isolates was monitored in B+N8P with 500 mg/L sulfolane. These cultures were inoculated with a 10% (v/v) inoculum of a stationary phase culture that had been grown in B+N8P with 500 mg/L sulfolane. Cultures were refed with aproximately 500 mg/L sulfolane once, then a 10% v/v inoculum was transferred to fresh medium. The OD_{600}, sulfolane concentration, and pH of the cultures were monitored regularly during the incubation period.

2.11.2.3 Effect of sulfolane analogs on aerobic pure cultures

The ability of pure, sulfolane-degrading cultures to utilize sulfolane in the presence of sulfolane analogs, and to utilize five sulfolane analogs (Figure 2.4) was examined by monitoring culture growth by OD_{600} and removal of sulfolane or sulfolane analogs by GC. The experimental protocol is described in Section 2.11.1.3.

2.11.2.4 Growth of E. coli K12 thiophene-degrading mutants on sulfolane

D. Clark (Department of Microbiology, Southern Illinois University, Carbondale, IL.) provided nine strains of *E. coli* K12 (Table 2.7). The *E. coli* strains were shipped to the laboratory by D. Clark on agar plugs and stored at 4°C until used. These plugs were used to inoculate LB and LB agar, modified with the appropriate antibiotics (MW strains: tet^R; NAR711: tet^R, amp^R; NAR820: tet^R, cm^R; NAR870: tet^R, cm^R, amp^R). Cultures of the *E. coli* mutants were grown in 50 mL M9 minimal salts medium (Sambrook et al., 1989) in 125-mL flasks, shaken at 200 rpm, 28°C. The C source added to the M9 medium was 0.4% (w/v) glucose, 0.4% glucose and 50 mg/L sulfolane, or 50 mg/L sulfolane. Each mutant was tested with each of the three C source combinations. Cultures which grew became visibly turbid. Sulfolane concentration was measured by GC.

2.12 Carbon and sulfur balance experiments

2.12.1 Method development

2.12.1.1 Initial serum bottle experiments

Experiments were designed to determine whether CO_2 was released during sulfolane degradation. The amount of O_2 required for sulfolane degradation was calculated from the following equation:

$$C_4H_8O_2S + 6.5O_2 + H_2O \rightarrow 4HCO_3^- + 6H^+ + SO_4^=$$

Based on the assumption that air contains 21% O_2 (Serway, 1986), at room temperature 117 L air woud provide 1 mol O_2, therefore a 158-mL serum bottle could contain up to 35 mL of a 4.17 mM (500 mg/L) sulfolane and allow sufficient O_2 for complete mineralization

Table 2.7. Strains of *Escherichia coli* K12 able to degrade thiophene, provided by D. Clark (Department of Microbiology, Southern Illinois University, Carbondale, IL.). Originally described in Juhl and Clark (1990).

strain identity	genetic characteristics of strain[a]
W1485	wild type
DC625	*gyr*A, *nal*A, *fad*R
NAR11	spontaneous *cys*$^+$ revertent of NAR10[b]
NAR711	pKA15[c] in *rec*A, srl::Tn10 from DC625
NAR820	*rec*A::cm, *thd*A+ Tn10 from SG20253[d] of NAR30[e]
NAR870	pKA15 in NAR820
MW90	zba::Tn10 *thd*A2 mutant of DC625/P1[f]
MW96	*fad*R+zcf::Tn10 mutant of NAR11/P1[f]
MW100	zba::Tn10 *thd*A3 mutant of DC625/P1[f]

[a] codes for genetic mutations are as follows: *gyr*A DNA gyrase, *nal*A nalidixic acid, *fad*R repressor of fatty acid degradative genes, *cys*$^+$ cysteine, *rec*A recombinase, cm chloramphenicol resistance, *thd*A regulatory gene for thiophene degradation, tet tetracycline resistance

[b] NAR10: first stage thiophene-degrading mutant of DC625, *thd*A1 (Abdulrashid and Clark, 1987)

[c] pKA15: plasmid construct containing the *thd*A gene on a 0.9 kb *Pst*I chromosomal fragment inserted in the pUC19 multiple cloning site (Alam and Clark, 1991).

[d] SG20253: zba300::Tn10

[e] NAR30: *thd*A1, *thd*D, *thd*C mutant of DC625 (Abdulrashid and Clark, 1987)

[f] P1: cotransduction with bacteriophage P1*vir* followed by selection for tet^R transductants (Miller, 1972)

of sulfolane. Acetate was also used as a C source; it was added at a concentration of 8.33 mM in order to provide the same number of moles of C as sulfolane.

2.12.1.2 Fermentor experiments

The production of CO_2 during sulfolane degradation by the isolate HPRK2-S28 was monitored in a 10-L culture in a 14-L fermentor. CO_2 was trapped in NaOH, then titrated. Air added to the fermentor was scrubbed to remove CO_2. A diagram of this equipment is shown in Figure 2.5. The fermentor was filled with 10 L $SO_4^{=}$-free medium. Supervision and set-up for the fermentor were carried out by R. Mah (Department of Biological Sciences, University of Alberta, Edmonton, AB).

The inoculum was prepared by growing 7.2 L of HPRK2-S28 in B+N8P with 500 mg/L sulfolane, and harvesting the cells by centrifugation (10 400 x g, 4°C). Cells were resuspended in $SO_4^{=}$-free medium, then centrifuged once more, as a wash step, then resuspended in 30 mL $SO_4^{=}$-free medium to inoculate the fermentor. The total wet weight of cells used as an inoculum was 1.92 g. At the time of inoculation, a final sulfolane concentration of 500 mg/L was added to the fermentor, and polypropylene glycol (1 mL) was added to reduce foaming. The air flow was set to a range between 1 L/min and 2 L/min, pH was maintained at 6.75, and the propeller speed was 200-210 rpm. Incoming air was scrubbed to remove CO_2 by passing through 1 M NaOH, then a cotton plug and self-indicating Drierite (W.A. Hammond Drierite Company, Xenia, OH) to remove airborne NaOH, and three consecutive traps filled with self-indicating Carbosorb soda lime (1.4 to 4 mm, BDH, Poole, GB), then was passed through a sterile filter before entering the fermentor. Samples were removed from a sterile sampling port to analyze the activity of the culture over time.

After the removal of sulfolane was complete, CO_2 in the NaOH traps was quantified by titration (Alef and Nannipieri, 1995). For this method, 5 mL of a stock solution of $BaCl_2$ (122.14 g $BaCl_2 \cdot 12H_2O$ per 1 L water) and a few drops of indicator (0.1 g phenolphthalein in 80 mL 60% v/v ethanol in water) were added to 25 mL NaOH from the CO_2 trap. Using a buret, 0.05 M HCl was added until the solution became colorless. A modified version of the equation described by Alef and Nannipieri (1995) for calculating CO_2 in a soil sample was used to calculate the CO_2 present in the NaOH trap: $CO_2\ (mg) = (Vo)(1.1)$

where Vo = the volume of HCl used in the titration and 1.1 = the conversion factor (1 mL 0.05 M NaOH = 1.1 mg CO_2).

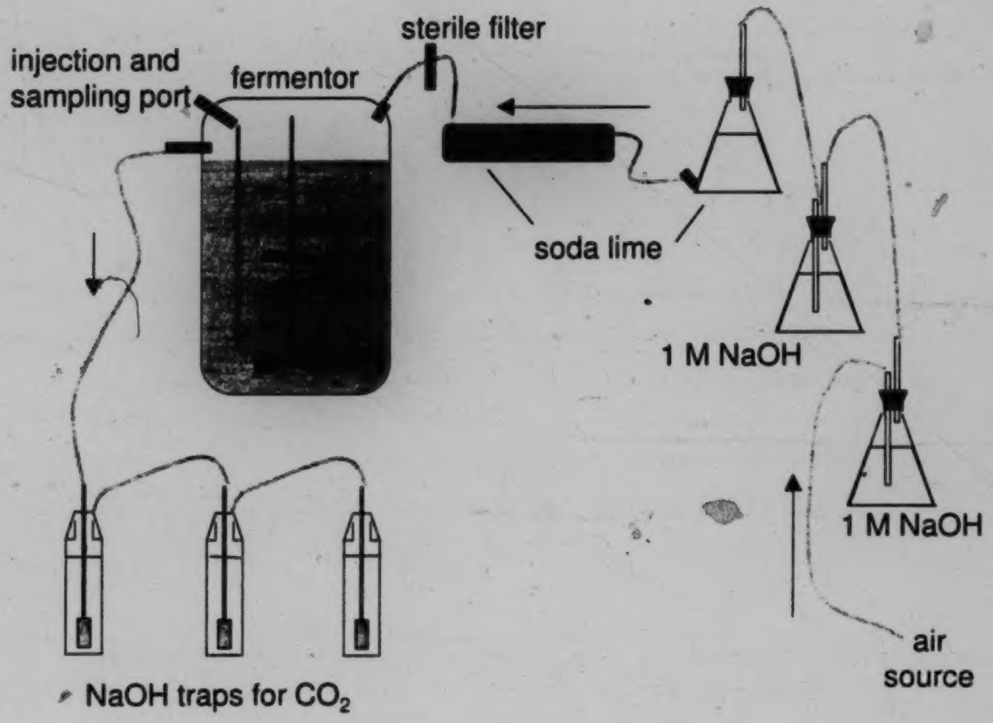


Figure 2.5. Diagram of the fermentor system for measuring CO_2 production from sulfolane degradation.

Biomass C generated was calculated by comparing the start time and end time wet weight of the biomass. A sample of the total fermentor culture was centrifuged in the same manner of the inoculum; total wet weight biomass generated in the fermentor was calculated from this sample. The wet biomass was transferred to a 500-mL round-bottom flask and freeze dried; this sample was analyzed for the N, C, H and S content (Analytical Service Laboratory, Department of Chemistry, University of Alberta, Edmonton, AB). This sample was used as a standard for biomass in later experiments measuring total organic carbon. Sulfate was calculated using the $BaCl_2$ turbidimetric method (Standard Methods, 1989), modified as described in Section 2.2.10.4. Sulfolane was analyzed by GC.

2.12.1.3 Shake-flask experiments

Initially, shake-flask C-balance experiments were done in 2-L sidearm flasks. The sidearm opening was sealed with a rubber septum; the neck of the flask was sealed with a sterile rubber stopper. Calculations for the amount of air space required for total sulfolane oxidation in a 400 mL culture (Section 2.12.1.1) indicated that the required headspace volume was 1 400 mL. The cultures were grown in $SO_4^{=}$-free medium supplemented with 500 mg/L sulfolane. Inoculum was HPRK2-S28, grown in B+N8P medium and washed in $SO_4^{=}$-free medium as described in Section 2.12.1.2. A total of 6 x 200-mL cultures were used as inocula. Six cultures were incubated; three viable to measure CO_2, two viable to measure $SO_4^{=}$ and biomass and one sterile control.

Samples analyzed for CO_2 were injected with [illegible]0 mL 1 M NaOH, in order to trap all the CO_2 present in the supernatant, shaken for 24 h, then 5-mL samples were removed and titrated (Section 2.12.1.2). Sulfolane was analyzed by GC. Biomass was harvested by centrifugation, and the supernatant was retained for $SO_4^{=}$ analysis.

Further shake-flask C-balance experiments were carried out in 500-mL Erlenmeyer flasks, each fitted with two glass sampling ports approximately 5 cm above the bottom of the flask. The sampling ports were sealed with rubber septa; the top of each flask was sealed with a rubber stopper. Each flask contained 200 mL $SO_4^{=}$-free medium, 1 drop of a saturated solution of bromthymol blue pH indicator in deionized, distilled water and either 200 mg/L or 500 mg/L sulfolane. Bromthymol blue was not added to all cultures as it interfered with total organic C analysis; instead the pH of cultures was monitored in 0.5-mL samples removed from the cultures. As sulfolane was degraded and the pH in each culture dropped, sterile 0.2 M NaOH was injected to return the culture pH to approximately 7. The mixed culture HM8-S28 was used as an inoculum. Samples were taken in 10-mL volumes, transferred to sterile 15-mL centrifuge tubes, and centrifuged in a clinical centrifuge (890 x g) for 10 min. The supernatant and cell pellet, resuspended in 10 mL $SO_4^=$-free medium were retained separately for total organic C analysis. As with the larger cultures, CO_2 was trapped with 1 M NaOH, then measured by titration.

2.12.1.4 Serum bottle method for carbon and sulfur balance

The amount of supernatant present in the initial design for the serum bottle method to determine a C and S balance was too small for accurate measurements. In 158-mL serum bottles, the headspace, filled with air, should support the complete mineralization of 16 mg sulfolane (360 mg/L) in a 50-mL culture, therefore sulfolane concentrations of 50, 100, 200 or 300 mg/L were used. Analysis of CO_2 by trapping in NaOH and titration was tedious, and errors may have occurred during the transfer of samples from the incubation vessel to the vessel for analysis. The serum bottle experiment system was therefore designed so that cultures analyzed for CO_2 production could be acidified in the serum bottle, then the headspace gas analyzed by GC. Replicate cultures were used to analyze CO_2, $SO_4^=$ and total organic C; the samples taken for C analysis were split into biomass and dissolved C fractions by centrifugation. Extra replicate cultures were incubated; these were used to monitor cell growth (by OD_{600}) and pH. Sulfate-free medium containing sulfolane was prepared and distributed into 158-mL serum bottles. Inocula were grown in $SO_4^=$-free medium for at least three transfers, then freshly grown culture was injected directly into serum bottles rather than prepared by centrifugation and washing.

Samples analyzed for the presence of CO_2 were acidified to pH ≤ 2 by injecting 0.1 mL 10 M HCl and shaking overnight on a rotary shaker in order to drive CO_2 into the headspace. Samples were stored at 4°C until the headspace gas was analyzed by GC. Samples analyzed for the presence of $SO_4^=$ were centrifuged; the supernatant was retained and filter sterilized (CoStar μStar LB 0.22 μm pore size CA filter), then stored at 4°C until analysis. Sulfate was analyzed using the $BaCl_2$ turbidimetric method (Standard Methods, 1989) modified for smaller sample size (Section 2.2.10.4) or by ion chromatography. Samples used to measure total organic C were centrifuged in 50-mL tubes at 12 100 x g for 20 min. Supernatants were removed and frozen at -20°C until analysis. Cell pellets were resuspended in 10 mL deionized, distilled water and frozen at -20°C until analysis. Whenever time course experiments were done, all samples were stored until the end of the experiment and analyzed simultaneously.

2.12.2 Sulfate release time course for mixed and pure cultures

The release of $SO_4^=$ during sulfolane degradation was monitored in triplicate 200-mL shake-flask cultures in $SO_4^=$-free medium. Cultures were supplemented with 300 or 500 mg/L sulfolane. Inocula were 10 mL of culture freshly grown in $SO_4^=$-free medium. As sulfolane was degraded and the cultures became acidic they were neutralized by the addition of sterile 0.2 M NaOH. Samples of approximately 12 mL were taken in 15-mL sterile centrifuge tubes, centrifuged at high speed in a clinical centrifuge (approximately 890 x g), filter sterilized, and stored at 4°C until the end of the experiment, when they were all analyzed simultaneously. $SO_4^=$ was measured using the $BaCl_2$ turbidimetric method (Section 2.2.10.4). Sulfolane was analyzed by GC and cell growth was measured by OD_{600}.

2.13 Attempts to identify metabolites from sulfolane degradation

A pure culture, WPW-S28, was selected for experiments to identify sulfolane metabolites. Two general approaches were taken: attempts to disrupt sulfolane-degrading genes, and attempts to identify the metabolites in culture.

2.13.1 Attempts to disrupt genes for sulfolane degradation in the bacterial isolate WPW-S28

A random transposon mutagenesis of WPW-S28 was carried out using the *E. coli* suicide vector pGS9 (Selvaraj and Iyer, 1983). The *E. coli* HB101 pGS9 strain was obtained from J. Foght (Dept. of Biological Sciences, University of Alberta, Edmonton, AB), and grown in LB medium containing 50 mg/L kanamycin and 100 mg/L chloramphenicol. WPW-S28 was grown in B+N8P medium containing 500 mg/L sulfolane. Overnight cultures of *E. coli* HB101 pGS9 and cultures of WPW-S28 that had just completed sulfolane removal from the medium were used for the mating experiments. *E. coli* HB101 pGS9 was mated with WPW-S28 using a modified version of the Eppendorf mating protocol described by Singh and Klingmüller (1985), a modified version of the filter mating protocol described by Selvaraj and Iyer (1983), or a modified version of the cross-streak protocol (Owen and Ward, 1985).

For Eppendorf matings, 1 mL WPW-S28 was mixed in a sterile Eppendorf tube with 0.15 mL *E. coli* HB101 pGS9, centrifuged at 14 000 rpm in an Eppendorf microfuge for 10 min, and all but approximately 0.1 mL of the supernatant was drained. This sample was incubated overnight at 30°C, then resuspended in 900 µL sterile B+N8P medium and 0.1-mL aliquots were spread on plates of selective B+N8P + glucose + kan medium, which would not support the growth of *E. coli*, but allowed WPW-S28 to grow if it had received the kan^R phenotype carried by pGS9. This selective agar was made up of B+N8P medium, 8 mg/L glucose, 50 mg/L kan and 15 g/L Bacto agar (Difco). Plates were incubated for approximately 2 wk before colonies developed.

Filter matings were carried out by mixing 9 mL WPW-S28 culture and 1 mL *E. coli* HB101 pGS9 culture in a 10-mL syringe and filtering the resulting cell suspension through a sterile 0.22 μm pore size nitrocellulose-containing filter (Millipore GS filter) in a re-usable filter casing (Millipore Swinnex-25 casing). The filter paper was then transferred to LB agar and incubated at 30°C overnight. Cells were resuspended from the filter in 10 mL sterile B+N8P medium, which was plated on selective B+N8P + glucose + kan medium (described above) at a 10^{-2} dilution and incubated for approximately 2 wk.

Cross-streaks of WPW-S28 and *E. coli* HB101 pGS9 were prepared by streaking parallel lines of a WPW-S28 culture approximately 1 cm apart on a LB agar plate, and streaking an *E. coli* HB101-pGS9 culture in similar parallel lines perpendicular to the lines of WPW-S28 (Owen and Ward, 1985). Plates were incubated for 1 to 2 wk, until the WPW-S28 streaks had grown. Cells were harvested from the regions where the two bacterial cultures crossed, then resuspended in sterile B+N8P medium, plated onto selective B+N8P + glucose + kan medium and incubated 1 to 2 wk.

After incubation of selective plates inoculated with the mating mixtures of *E. coli* and WPW-S28, isolated colonies were transferred to B+N8P + glucose + kan selective plates (50 colonies per plate) and incubated a second time. When these colonies had grown (approximately 2 wk) the patch plates were replica plated onto B+N8P + glucose + kan plates and PAT medium + kan plates and incubated for another 2 wk. Colonies that did not appear to degrade sulfolane were streaked onto PAT medium plates; those which continued to appear unable to degrade the compound were examined to determine that they still phenotypically resembled WPW-S28, and transferred to liquid medium (10 mL B+N8P medium containing 500 mg/L sulfolane) to determine whether they could degrade sulfolane. A Birnboim alkaline lysis plasmid preparation (Sambrook et al, 1989) was carried out on colonies that still appeared to be unable to degrade sulfolane, to ensure that the Tn10 cassette bearing the kan^R phenotype had integrated into the WPW-S28 chromosome.

2.13.2 Washed cell experiments

Cultures of WPW-S28 were prepared for washed cell experiments by inoculating 200 mL B+N8P medium containing 500 mg/L sulfolane in 500-mL Erlenmeyer flasks, each with 10 mL of a culture that had recently completely removed sulfolane from a similar medium. Within 24 h of complete sulfolane removal from the cultures, cells were harvested, washed with, and resuspended in 0.05 M KH_2PO_4 at 25% of the original culture volume, then distributed into 158-mL serum bottles.

Washed cell cultures consisted of 25 mL freshly grown WPW-S28 resuspended in 0.05 M KH_2PO_4, in 158-mL serum bottles sealed with butyl rubber stoppers. Cultures were incubated shaking at 200 rpm, 28°C. Sulfolane was added to each culture at the start time; as some samples were harvested within 30 min these start times varied depending on the experiment. The sulfolane concentration in the experimental samples was monitored using two replicate cultures designated 'test' cultures, which were sampled regularly during the incubation period.

At sampling times, cultures were harvested and supernatants were prepared for derivatization of compounds that might have been released from sulfolane degradation. Cultures were centrifuged for 20 min at 7 710 x g. The culture supernatants were filter sterilized with a 0.22 μm pore size filter; cell pellets were resuspended in 5 mL deionized, distilled water and both samples were frozen at -20°C.

2.13.3 Preparation of samples for derivatization

The C and S balance experiments (Section 2.12) demonstrated that not all the C and S from sulfolane were converted to CO_2, $SO_4^{=}$, and biomass. The remaining compounds were predicted to be small and polar and were likely to be more easily detected if they were derivatized prior to analysis; therefore three different derivatization methods were tested to determine whether this treatment would assist in the detection of sulfolane degradation metabolites.

2.13.3.1 Preparation of cell cultures for derivatization by freeze drying

For derivatization of compounds present in the serum bottle experiments (Section 2.12.4), triplicate cell cultures that had each been treated with 0.1 mL 10 M HCl per were combined and freeze dried in 250-mL round-bottom flasks. The freeze-dried compounds were dissolved in diethyl ether and derivatized in the round-bottom flasks. For derivatization of compounds present in the washed cell experiments, culture supernatants were thawed and treated with either 1 mL 10 M HCl or 1 mL 10 M NaOH, then freeze dried. In three separate experiments, freeze dried materials were: dissolved in acetonitrile, transferred to 4-mL glass vials and derivatized, or dissolved in water, transferred to 4-mL dram vials, dried under N_2, dissolved in acetonitrile and derivatized, or derivatized in acetonitrile in the round-bottom flasks.

2.13.3.1 Preparation of a basic ion exchange resin for trapping polar compounds in culture medium

Rohm and Haas basic ion exchange resin Amberlyst A26 was obtained (Aldrich). The resin was activated by placing 10 g resin in a Millipore 50-mL Steriflip disposable vacuum filtration tube system, washing four times in 30 mL deionized, distilled water (water is removed using the vacuum filtration system), washing for 5 min once in 30 mL 0.1 M $NaHCO_3$ and draining to dryness. Activated resin was stored in a sealed disposable 50-mL centrifuge tube until used. Approximately 1 g of activated ion exchange resin was added to medium containing compounds of interest to test the ability of the resin to trap polar metabolites. After incubation, the resin was collected from the medium by vacuum filtration, using a Millipore type HV 0.45 μm filter. Beads were washed five times with deionized, distilled water, transferred to a 50-mL round-bottom flask and derivatized with methyl iodide (iodomethane) in benzene.

2.13.4 Derivatization methods

2.13.4.1 Diazomethane

Diazomethane was generated using the method of Gieg (1996). A Millimole generator apparatus (Wheaton) was cleaned with acetone and dichloromethane. The inner tube of the apparatus was used to dissolve 0.132 g 1-methyl-3-nitro-1-nitrosoguanidine (97%, Aldrich) in 0.5 mL deionized, distilled water, then sealed with a Teflon stopper and screw cap. The outer tube of the generator was chilled on ice, then filled with 3.0 mL chilled diethyl ether, clamped to the inner tube and submerged in an ice bath. The reaction was carried out by slowly injecting 0.6 mL 5 M NaOH through the Teflon stopper into the 1-methyl-3-nitro-1-nitrosoguanidine solution; diazomethane generation occurred over approximately 45 min and the gaseous compound was trapped in the chilled diethyl ether. Diazomethane was generated fresh for each reaction, added to freeze-dried samples dissolved in diethyl ether in 0.5-mL quantities, and allowed to react for 1 h. Samples were then collected in diethyl ether, transferred to 4-mL glass vials, dried under N_2 and redissolved in dichloromethane for GC analysis.

2.13.4.2 Methyl iodide (iodomethane)

Derivatization of freeze-dried samples with methyl iodide was carried out in round-bottom flasks attached to a reflux apparatus cooled with water, and heated with a heating mantle controlled by a rheostat. Samples were placed in each flask, along with 6-10 Teflon/halon Chemfluor boiling chips (Chemware, Chemplast Inc, Wayne, NJ), 500 mg (220 μL) methyl iodide (iodomethane, Aldrich) and 3 mL 99.9% pure HPLC grade benzene (Sigma/Aldrich). Samples were allowed to reflux for approximately 18 h, then were cooled to room temperature. The samples dissolved in benzene were transferred into 4-mL glass vials, dried under N_2 and dissolved in dichloromethane for GC analysis.

2.13.4.3 N,O-bis(trimethylsilyl)acetamide (BSA)

Freeze-dried samples were dissolved in acetonitrile in round-bottom flasks or transferred to 4-mL glass vials for derivatization. For a sample dissolved in 0.5 mL acetonitrile, 250 μL BSA was added. Samples were sealed, mixed well, and heated to 70°C for 15 min. After cooling to room temperature, the samples were dried under N_2 and dissolved in dichloromethane for GC analysis.

2.14 Identification of pure culture WPW-S28

2.14.1 Sequence of 16S rRNA

2.14.1.1 Purification of genomic DNA from WPW-S28

Genomic DNA was prepared according to the method of Hopwood et al. (1985), from an overnight culture of WPW-S28 grown in 200 mL LB medium at 28°C, shaking at 200 rpm in the dark. The culture was centrifuged in 50-mL centrifuge tubes at 4°C, 7 710 x g for 30 min. Supernatant was decanted and cells were resuspended in 1 mL TES buffer per tube (4 total), divided into eight sterile 1.5-mL Eppendorf tubes (700 μL/tube), and centrifuged in an Eppendorf microcentrifuge at 14 000 rpm for 5 min. TES buffer consisted of 30 mM tris(hydroxymethyl)aminomethane (Tris-OH, Aldrich), 5 mM EDTA and 50 mM NaCl in deionized, distilled water. The supernatant was discarded and pellets resuspended in 600 μL TES buffer per tube, to which 150 μL of a fresh 5 mg/mL lysozyme (Sigma) solution (in TES buffer) was added. Samples were incubated at 37°C for 30 min, then 45 μL 10% w/v SDS in TES buffer was added, mixed by inversion, and incubated a second time at 37°C for 10 min before the addition of 2.5 μL 10 mg/mL RNase A (Sigma), and a third incubation at 37°C for 45 min. At this time 25 μL 25 mg/mL Pronase E (Sigma) was added to samples and they were incubated overnight at room temperature. Samples were then transferred to four 15-mL disposable centrifuge tubes, extracted with 1 volume of a neutral phenol/chloroform solution (1:1 v/v), transferred to fresh 15-mL centrifuge tubes, layered with 95% ethanol (approximately 10 mL) chilled to -20°C, and 50 μL 3 M sodium acetate, and mixed by inversion. DNA was spooled onto a glass rod, dipped in a tube filled with 70% ethanol chilled to -20°C, then transferred to a 15-mL centrifuge tube. This sample was resuspended in 10 mL sterile deionized, distilled water, transferred to a 50-mL disposable centrifuge tube, and diluted for analysis. DNA preparations were analyzed on a 0.5% agarose gel; those which showed the least cleavage of chromosomal DNA were retained for PCR experiments.

2.14.1.2 Primer design

Two primers were designed for generating a PCR fragment encompassing most of the 16s rRNA sequence present in WPW-S28. These primers were also used for the initial portion of the sequencing reaction. The forward primer, 8^{F}, encompassed the forward amplification primer sequence 16F27 from Hauben et al. (1997): $^{5'}$AGAGTTGATCATGGCTCAG$^{3'}$ and a sequence designed by P. Beatty (Dept. of Biological Sciences, University of Alberta, Edmonton, AB) that contained sites for the restriction enzymes *Sac*1, *Eco*R1 and *Xba*: $^{5'}$GAGCTCTAGAATTC$^{3'}$. The reverse primer sequence (1403^{R}) encompassed the reverse amplification primer sequence 1387R from Marchesi et al. (1998): $^{5'}$CGGAACATGTGAGGCGGG$^{3'}$ and the reverse of the restriction enzyme site sequence provided by P. Beatty. The restriction enzyme sites were added to the PCR primers so that if it were necessary to clone the PCR fragment into a plasmid for further genetic analyses, there would be an easy means to prepare it. The primer sequences were compared with those for the *E. coli* 16S rRNA sequence (Carbon et al., 1979); the primers described above extend from nucleotides 8 to 27 in a forward direction and from nucleotides 1403 to 1386 as a reverse complement to the reported sequence, which is a total of 1541 nucleotides in length.

The amplification primers described above were used as sequencing primers to obtain the end portions of the WPW-S28 16S rDNA sequence. Other sequencing primers were designed based on a series of 10 primers described by Hauben et al. (1997). Modifications to the suggested sequences were made based on the sequences for WPW-S28 and *P. polymyxa* which were obtained using the amplification primers. A summary of these primers is shown in Table 2.8. Sequencing reactions did not occur with some of the primers that were tested, and with others the sequence obtained for WPW-S28 was of poor quality and was therefore discarded. In some cases, the sequence for a similar region of the positive control for this experiment, *P. polymyxa*, which was generated in tandem with that of WPW-S28, was successful. Sequencing primers that did not produce acceptable results are not reported in Table 2.8.

2.14.1.3 Generation of 16S rDNA PCR fragment from WPW-S28 genomic DNA

A PCR protocol that produced the maximum amount of 16S rDNA from WPW-S28 genomic DNA, using the primers 8^{F} and 1403^{R} (Table 2.8), was developed. This

Table 2.8. Amplification and sequencing primers used to determine the 16S rRNA sequence of WPW-S28. Primers were based on similar sequences reported by Hauben et al. (1997).

primer name	primer sequence	application
8^F	$^{5'}$GAGCTCTAGAATTCAGAGTTTGATCATGGCTCAG$^{3'}$	amplification
704^F	$^{5'}$TGTGTAGCGGTGAAATGCGTAGA$^{3'}$	sequencing
1176^F	$^{5'}$AGGAAGGGGGGGAGGAGGT$^{3'}$	sequencing
358^R	$^{5'}$CCCACTGGTGCCTCCCGTA$^{3'}$	sequencing
1106^R	$^{5'}$CGCCCTTTTCGGGACTTAACCC$^{3'}$	sequencing
1403^R	$^{5'}$TCGAGCTCTAGAATTCGGGCGGTGTGTACAAGGC$^{3'}$	amplification

Table 2.9. Temperature program for amplification of the 16S rDNA fragments of WPW-S28 and *P. polymyxa* by PCR.

step	protocol
1	5 min 94°C
2	30 s 94°C denaturation of DNA primers and template
3	30 s 55°C annealing of DNA primers and templates
4	1.5 min 72°C extension of new DNA strands
5	repeat steps 2 through 4, 29 times
6	5 min 72°C extension of new DNA strands
7	4°C stop PCR reaction and refrigerate DNA product
8	end

protocol was used to generate DNA fragments for sequencing in order to determine the 16S rRNA sequence of WPW. The concentration of genomic and primer DNA was determined spectrophotometrically using the A_{260} of a DNA solution in water. PCR reactions were carried out in an MJ Research Minicycler. The temperature program was designed based on those of Hauben et al. (1997) and Marchesi et al. (1998), with modifications developed experimentally to suit WPW-S28 and *P. polymyxa* gene amplification, and is shown in Table 2.9.

PCR generation of the 8^F-1403^R PCR fragment for sequencing was carried out in a set of 16 identical reaction tubes. Reactions contained: 1 µL 30 ng/µL WPW-S28 genomic DNA, 0.67 µL each of 30 pmol/µL primer 8^F and 30 pmol/µL primer 1403^R, 0.4 µL 25mM dNTP mix (25 mM each of dATP, dCTP, dGTP and dTTP, Boeringher Mannheim), 5 µl 10X PCR buffer, 0.75 µL 0.1 M $MgCl_2$, 40.51 µL sterile deionized, distilled water and 1 µL 5 units/µL taq DNA polymerase (purified by A. Hashimoto, Dept. of Biological Sciences, University of Alberta, Edmonton, AB). PCR buffer (10x) contained 0.5 g KCl, 8.48 g tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl, pH 8.8), 1 mL 1% Triton X-100 per 100 mL, in deionized, distilled water. After preparation, a small amount of sterile mineral oil was put in each tube and tubes were centrifuged for a few seconds to bring the solution and oil to the bottom. The taq DNA polymerase was not added to the reaction mix until the PCR program had run for approximately 2 min; at this time the PCR tubes were opened, enzyme was added beneath the mineral oil layer, and the program was allowed to continue. This 'hot start' procedure was used in order to decrease possible nonspecific product formation. Positive control (*P. polymyxa*) PCR reactions consisted of the same ingredients as the WPW-S28 amplification mixture, except that 1.2 µL of 25 ng/µL *P. polymyxa* genomic DNA was substituted for the 1.0 µL 30 ng/µL WPW-S28 genomic DNA, and that due to this volume change only 28.25 µL sterile deionized, distilled water was added to each reaction. For PCR reactions used to test the ability of the sequencing primers to interact with WPW-S28 genomic DNA, the primers of interest were substituted for primers 8^F and 1403^R, at the same concentrations as these primers. Each test reaction was run with the sequencing primer of interest acting as the amplification primer in one direction, and either the primer 8^F or the primer 1403^R as the second direction amplification primer in the test reaction.

2.14.1.4 Purification of fragment

The approximately 1400 base pair (bp) DNA fragment produced by PCR amplification of the WPW-S28 16S rDNA gene was purified for sequencing. After PCR, the aqueous portion of each reaction tube was transferred to a sterile 1.5-mL Eppendorf tube; 8 PCR reactions were collected in each 1.5-mL tube. To remove any remaining mineral oil, each sample was extracted with an equal volume (400 µL) neutral phenol/chloroform (1:1 v/v) solution. The aqueous layers were transferred to clean tubes and precipitated with 800 µL 95% ethanol at -20°C for at least 30 min. Samples were resuspended in a total volume of 240 µL sterile deionized, distilled water and mixed with 40 µL 5x loading buffer (Sambrook et al., 1989). The DNA fragments representing the 16S rDNA PCR product were separated on a 0.8% ultrapure agarose (ICN, Aurora, OH) gel run at 100V (approximately 5 V/cm).

A DNA fragment was detected at approximately 1.4 kilobases (kb); this was excised with a razor and removed from the agarose using the Geneclean® kit (BIO 101, Vista, CA). For this procedure, agarose gel bands approximately 1.5 x 0.5 x 0.5 cm were placed in sterile 1.5-mL Eppendorf tubes and dissolved in 900 µL Geneclean® NaI solution at 55°C for 10 min. DNA was extracted onto glass beads by adding 10 µL Geneclean® glass milk solution, mixing by inversion, incubating at room temperature for 10 min, centrifuging at maximum speed for 5 s and discarding the supernatant. The glass bead and DNA pellet was washed three times by resuspending in 300 µL Geneclean® New Wash buffer, centrifuging 5 s and discarding the supernatant. After the final wash step, the pellet was air dried for 10 min and resuspended in 20 µL sterile deionized, distilled water by adding a 10µL portions of water, resuspending the glass beads, warming to 55°C for 10 min, then centrifuging 30 s and collecting the water two times. The DNA concentration was measured by fluorometry; if the sample was not concentrated enough for sequencing (200 µg/mL) then the remaining DNA preparation was precipitated by adding 60 µL 95% ethanol, chilling to -20°C for at least 30 min, centrifuging 10 min, discarding the supernatant and air drying the DNA pellet, which was resuspended in the appropriate amount of sterile deionized, distilled water to provide a sample for sequencing. Samples were checked by electrophoresis on a 0.8 % agarose gel as well as measuring their DNA concentration by fluorometry.

2.14.1.5 Sequencing of the 16S rDNA gene fragments

The 1400 bp DNA fragment generated by PCR of WPW-S28 or *P. polymyxa* genomic DNA using primers 8^F and 1403^R was sequenced by P. Murray (Dept. of Biological Sciences, University of Alberta, Edmonton, AB). Samples were submitted for sequencing in the form of PCR product (template) as 5 µL of a 200 µg/mL solution in sterile deionized, distilled water and primers (each separately at a concentration of 3 pmol/μL in sterile deionized, distilled water).

2.14.1.6 BLAST search protocol

The WPW-S28 16s rRNA sequence was submitted to the basic local alignment search tool (BLAST) sequence alignment system. This search tool was developed by the National Center for Biotechnology Information (USA). The BLAST search tool provides a sequence database for DNA, RNA and proteins (Peruski and Peruski, 1997). The web site for this is http://www.ncbi.nlm.nih.gov, and the reference for its use is Altschul et al. (1997). An advanced ungapped BLAST search with an expect value of 10 was used. A total of 50 identities were checked to ensure that the sequences the WPW-S28 sequence was compared with were > 1000 bp in length.

2.14.1.7 Ribosomal database project search protocol

The WPW-S28 16s rRNA sequence was submitted to the Ribosomal Database Project (RDP) for sequence alignment and for the generation of a specific phylogenetic tree (Maidak et al., 1997). A generalized tree for the organism was obtained, then a specific tree was designed. WPW-S28 was identified as a member of the β subgroup of purple bacteria (Woese, 1987), therefore organisms from the α, β and γ subgroups were used in the design of the tree. The web site for the RDP was formerly http://rdpwww.life.uiuc.edu/; the new site is http://www.cme.msu.edu/RDP. Both were used for this project.

2.14.2 Biochemical tests for identification of WPW-S28

2.14.2.1 API 20E and API 20NE identification tests

API 20E and API 20NE identification tests (bioMérieux, Marcy-l'Etoile, FR) were used for four sulfolane-degrading isolates: WPW-S28, WPU-S28, WPS1-S28 and WPS2-S28. Each culture was prepared for the test by incubation for approximately 1 wk on LB agar, to produce colonies that were used to inoculate the tests. Both tests were inoculated as the instructions that come with the kits described. In all cases, no growth occurred until 48 h after inoculation; at this time the test results were interpreted using the API manuals. In no cases did the biochemical identification profile for the test organism match exactly with a known organism in the manuals; the identities reported are those of the closest possible matches.

2.14.2.2 Growth of WPW-S28 on selective and differential media

Both selective and differential media were prepared to test the bacterial isolate WPW-S28. These media were all prepared using the instructions given in the Difco

Manual (1984); interpretations of the results were also carried out using the Difco Manual as a reference. The following media were prepared: bile esculin agar, DNase test agar, eosin methylene blue agar, lactose broth, MacConkey agar, sheep blood agar (provided by A. Szenthe, Dept. of Biological Sciences, University of Alberta, Edmonton, AB), mannitol salt agar, *Pseudomonas* isolation agar, *Salmonella/Shigella* agar, Simmon's citrate agar, starch agar, triple sugar iron agar slant and xylose lysine (XLD) agar. Each medium was inoculated with WPW-S28 or with *P. polymyxa* (used as a positive control organism), and incubated at room temperature until cell growth had occurred or until the incubation had proceeded for long enough that a lack of growth was deemed to mean an inability of the organism to grow on the substrate. Positive control media for growth of WPW-S28 were LB agar and PAT medium.

2.14.2.3 Cell stains of WPW-S28

Gram stains were done using the Bacto 3-step Gram stain kit (Difco). This kit works in a similar manner to regular Gram staining procedure, except that no water wash step is required. Cells were taken from both liquid culture and agar plates for Gram staining.

Flagellar stains were carried out by M. Hicks (Dept. of Biological Sciences, University of Alberta, Edmonton, AB) following a modified version of the Gray staining method Mayfield and Inniss, 1977). *P. polymyxa*, which possesses a number of peritrichous flagella, was used as a positive control for this stain.

3 RESULTS AND DISCUSSION

3.1 Development of an aqueous injection GC method for detection of sulfolane

At the outset of this project, the GC method for detection of sulfolane involved extracting the compound from soil or culture supernatant into dichloromethane, with dibenzothiophene as an internal standard. Relatively large (20-mL) samples of culture supernatant were required for each extraction, and these extractions were time consuming. The time required for GC analysis was also a drawback; each sample took over 28 min to elute (Figure 3.1A). With this method, frequent monitoring of any cultures was not possible, and monitoring more than a few cultures at a time involved a large effort. In order to simplify the analysis of sulfolane in samples, a direct aqueous injection method for GC analysis was developed. Sulfolane is soluble in water at concentrations greater than 300 g/L (Merck index, 1968). In contrast, sulfolane was not very soluble in dichloromethane, which is much less polar than water. The advantages of using aqueous GC injections were therefore two-fold; sulfolane recovery was likely to improve, and no extraction of samples would be necessary.

The GC method was based on that used by Novotny et al. (1977). A similar injection procedure was done with sulfolane dissolved in water (Section 2.2.1.1). Once sulfolane detection was established, oven temperatures were tested to optimize the peak shape isothermally; for a 2 m column 210°C gave the best results (Figure 3.1B). Sulfolane peak areas were reproducible over a concentration range of 1-1000 mg/L.

Few peaks, if any, appeared between the solvent peak and the sulfolane peak. In order to decrease the retention time for sulfolane and to increase the number of samples that could be analyzed daily, the column length was changed to 1.3 m. At this time, a 15 cm 'guard column' was attached to simplify cleaning and/or replacement of the front end of the column. These modifications to the GC column resulted in poor elution of sulfolane and separation of the sulfolane peak from the solvent peak at 210°C; by changing the oven temperature to 200°C this problem was solved. Sulfolane eluted between 4 and 5 min with this system (Figure 3.1C).

A different GC method for analyzing sulfolane was developed for the eventuality that analysis of the derivatization products of potential sulfolane metabolites might require the use of a GC with both a FID and a FPD. The column used was restricted to organic solvents. Sulfolane was dissolved in dichloromethane and injected in this GC; the resulting chromatograms for the FID and FPD are shown in Figure 3.1D.

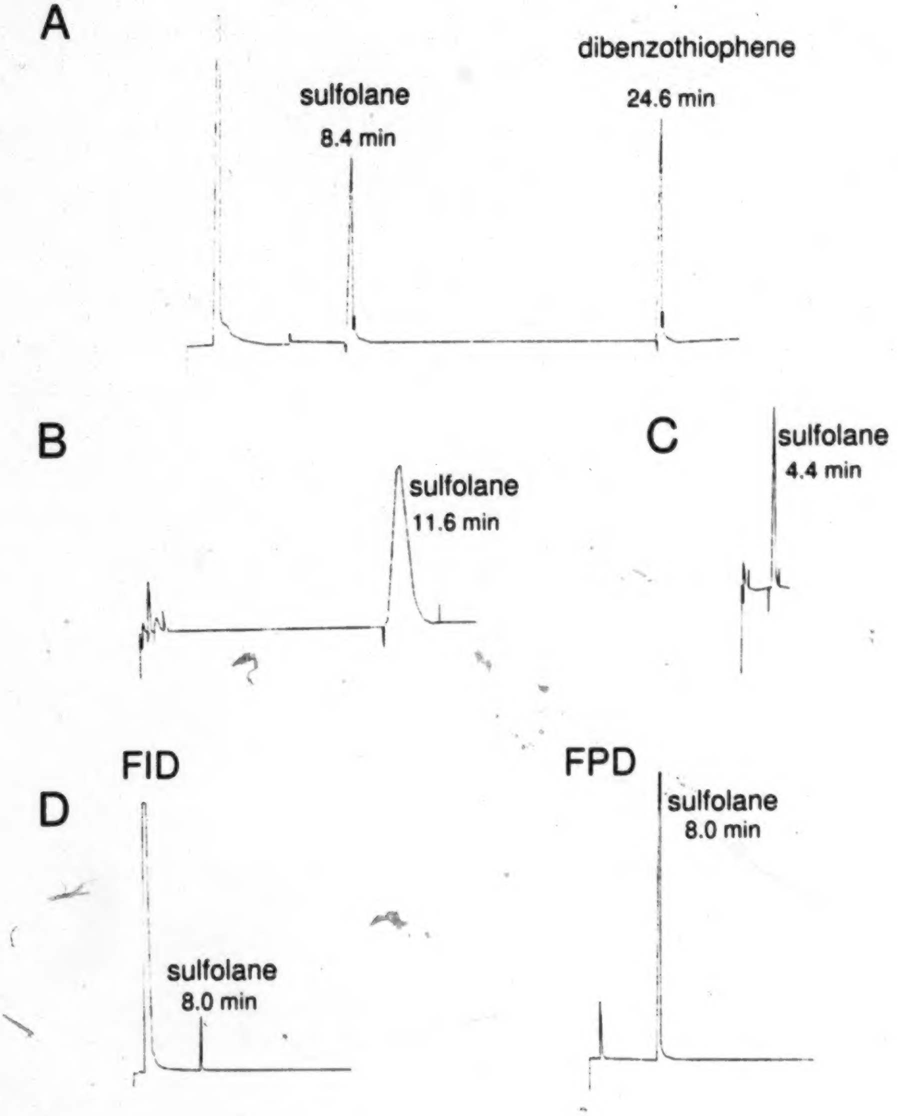


Figure 3.1. Chromatograms of sulfolane produced by different GC methods for detection. A. Sulfolane extracted into dichloromethane, analyzed on a DB-5 column. B. Sulfolane by direct aqueous injection into a 2 m long column. C. Sulfolane by direct aqueous injection into a 1.3 m long column. D. Sulfolane dissolved in dichloromethane, and injected into a GC equipped with a DB-5 column, a FID and a FPD.

3.2 Project to screen contaminated aquifer sediments and uncontaminated aquifer sediments and soils for sulfolane degradation potential

Some of the work described in Sections 3.2.1 and 3.2.2.2 was published by Greene et al. (1998). Some of the work described in Section 3.2.2.1 was published by Gieg et al. (1998). All the previously published research is presented in this thesis with the full knowledge and permission of all the authors listed on the published articles.

3.2.1 Aerobic shake-flask cultures

Incubation of aerobic shake-flask cultures was carried out at 8°C, typical summer groundwater temperature in Alberta, and 28°C, to increase biodegradation rates. These experiments were designed to screen contaminated aquifer sediments for their sulfolane degradation potential. Cultures were supplied with N and P, and were shaken to increase the diffusion of O_2 and nutrients in the medium and sediment inoculum. Shake-flask methods are described in Section 2.7.1.1, soils in Section 2.1 and Appendix A.

Some samples were inoculated with materials that had not been previously exposed to sulfolane. This portion of the experiment was carried out to determine whether exposure of a site to sulfolane resulted in the enrichment of a community capable of degrading the compound. Bridié et al. (1979) did not observe sulfolane degradation by a previously unexposed activated sludge inoculum over a 5 d experiment, whereas Chou and Swatloski (1983) observed sulfolane degradation by a similar inoculum after a lag time of approximately 5 d. Chou and Swatloski (1983) observed near stoichiometric release of $SO_4^=$ from sulfolane. Although this might have been a valuable measure of sulfolane degradation in the soil slurry cultures, because of the high concentrations of solids in the aerobic slurry microcosms, no attempt was made to detect $SO_4^=$. Aerobic sulfolane degradation occurred in all soils and sediments tested.

Aerobic degradation of sulfolane by the microorganisms in the contaminated aquifer sediment from the site 1 was observed within 0.7 d and 1.3 d, at 28°C and 8°C respectively (Figure 3.2A, B). The microcosms were refed with 200 mg/L sulfolane, resulting in its removal from the 28°C cultures within 0.4 d, and in the 8°C cultures, within 4 to 5 d. The lag period for the refed 28°C cultures was not detectable; for the 8°C cultures there was a 2 h lag period before sulfolane degradation commenced (data not shown). No loss of sulfolane from the sterile control cultures was observed during the course of these experiments (Figure 3.2A, B).

Aerobic degradation of sulfolane by the microorganisms in the contaminated aquifer sediment from site 2 was observed at 28°C after a lag period of just over 1 d, and

8°C after a lag period of 8 to 10 d (Figure 3.2C, D). The 28°C cultures were refed sulfolane, and removed the compound within 17 h after refeeding. The 28°C microcosms were refed a second time; this substrate was completely removed within 7 h. No significant lag period was observed after the 8°C microcosms were refed, and both responded in a similar fashion. Virtually all of the sulfolane was removed within 1 d. No loss of sulfolane from any of the sterile control cultures was observed during this time (Figure 3.2C, D).

Aerobic degradation of sulfolane by the microorganisms in the contaminated aquifer sediment from site 3 was observed at 28°C after a 1- to 1.3-d lag period, and at 8°C after approximately 2 d (Figure 3.2E, F). The 28°C cultures were refed sulfolane at 6 d, and this substrate was completely removed within 1 d of refeeding. The 8°C cultures were refed at 18 d, and sulfolane was removed within 3 d of refeeding, with no lag time observed. Sulfolane concentrations remained constant in both sterile control cultures during the experiment (Figure 3.2E, F).

Shake-flask cultures containing three uncontaminated soils were studied to compare their sulfolane biodegradation potential with those of the contaminated soils. One was an aquifer sediment from an uncontaminated region at site 2 (Table 2.1), the others were a greenhouse soil and a humus-rich soil. These cultures were incubated at 28°C as it was anticipated that biodegradation at 8°C would be much slower. The two cultures from site 2 degraded sulfolane completely in 8 or 13 d, after lag times of 7.5 or 9 d, respectively (data not shown). Greenhouse soil cultures showed lag times of 7 and 10 d, with the total removal of sulfolane occurring after 10 or 13 d in the two cultures, respectively (data not shown). Cultures inoculated with the soil high in humic material showed similar results, with lag times of 11 and 12 d. Sulfolane was completely removed from both these cultures by 14 d (data not shown).

The lag times for cultures that contained the uncontaminated materials incubated at 28°C were 7- to 17-fold longer and much more variable than those for cultures inoculated with contaminated materials (Table 3.1). These results clearly demonstrated that previous exposure to sulfolane enriches a microbial community so it can more rapidly begin to degrade this compound.

Gieg et al. (1998) found lag times of 95 to 120 h for aerobic DIPA biodegradation at 28°C in microcosms that contained these three uncontaminated soils. The 172 to 278 h lag times for sulfolane biodegradation (Table 3.1) show that sulfolane was more resistant to biodegradation by microbial communities in uncontaminated soils than DIPA.

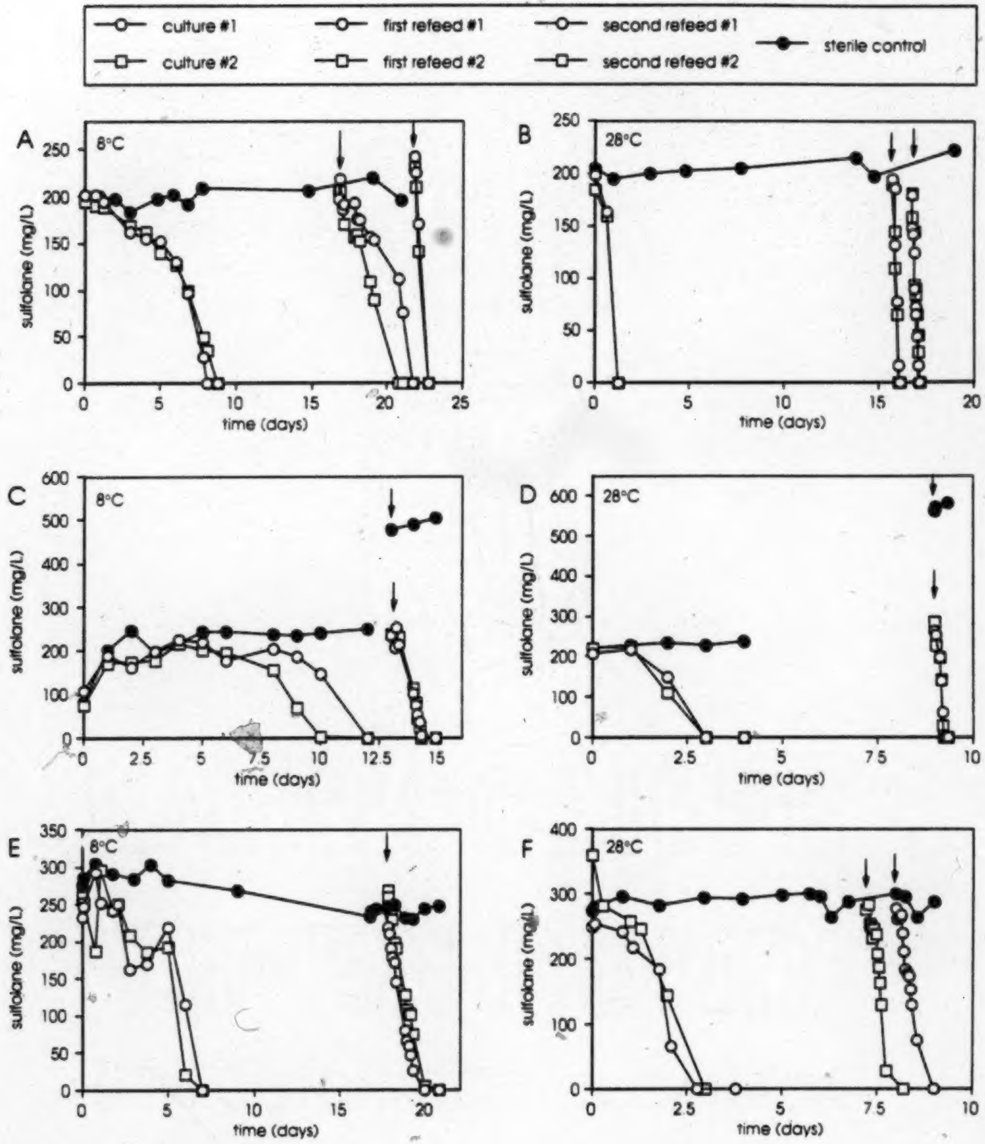


Figure 3.2. Loss of sulfolane from duplicate aerobic cultures inoculated with sediment and incubated at 8°C or at 28°C. A. Site 1 8°C. B. Site 1 28°C. C. Site 2 8°C. D. Site 2 28°C. E. Site 3 8°C. F. Site 3 28°C. Times at which cultures were refed with sulfolane are marked by arrows.

Table 3.1. The lag time, the kinetic order of degradation and biodegradation rate for sulfolane in the shake-flask cultures inoculated with sediment materials from sites 1, 2 and 3 or with uncontaminated sediment from site 2 or uncontaminated soil samples. Zero-order rates of degradation are given for the purpose of comparison. Average values for lag times and degradation rates are given; the individual values are shown in brackets.

soil or sediment inoculum in culture	incubation temperature	lag time (h)	kinetic order	zero-order rate of degradation (mg/L/h)
site 1 contaminated	8°C	30 (30-30)	zero	1.3 (1.2, 1.3)
site 2 contaminated	8°C	180 (144-216)	zero	2.4 (2.0, 2.7)
site 3 contaminated	8°C	50 (50-50)	zero	1.9 (1.7, 2.1)
site 1 contaminated	28°C	16 (16-16)	zero	6.4 (6.1, 6.6)
site 2 contaminated	28°C	24 (24-24)	unclear[a]	4.6 (4.6, 4.6)
site 3 contaminated	28°C	29 (26-31)	zero	4.9 (4.6, 5.2)
site 2 uncontaminated	28°C	200 (187-212)	zero	2.9 (1.5, 1.6, 5.7)
greenhouse soil	28°C	269 (260-278)	unclear[a]	3.9 (3.7, 4.0)
humus rich soil	28°C	208 (172-205)	zero	4.9 (2.8, 7.0)

[a] One replicate microcosm displayed zero-order kinetics, the other first-order kinetics.

Sulfolane is a S-containing cyclic compound, and is therefore likely to be more resistant to biodegradation than DIPA, a secondary amino alcohol.

In order to better predict the microbial contribution to sulfolane attenuation at the contaminated sites surveyed, the biodegradation rate and kinetic order of degradation were estimated. It should be noted that only simple kinetic models were considered for this analysis. These models for substrate disappearance, which include zero-order and first-order kinetics, are summarized by Alexander (1994). The kinetics and rates of sulfolane degradation were determined by comparing zero-order and first-order plots for the data points describing sulfolane loss from the cultures (Metcalf and Eddy, 1979). A sample of these graphs is shown in Appendix B; the kinetics data are summarized in Table B1.

Kinetic analyses indicated that sulfolane degradation was more accurately described by zero-order than first-order kinetics (Table B1). The zero-order rate constants determined from each set of duplicate cultures were generally similar; the largest difference between two values was 0.7 mg/L/h (site 2 at 8°C). For each of the contaminated sites, the rate of degradation in the microcosms incubated at 28°C was greater than 8°C: 4.9-fold for site 1, 1.9-fold for site 2 and 2.6-fold for site 3 (Table 3.1). After the microcosms had been refed, the rates of sulfolane biodegradation increased. The smallest increase (1.4-fold) was observed in the site 1 cultures incubated at 8°C, whereas the greatest increase (9.6-fold), was seen in the site 2 cultures incubated at 28°C (Table 3.2).

The zero-order rates for sulfolane biodegradation demonstrated by uncontaminated soil or sediment cultures were 2.9 to 4.9 mg/L/h; two of the three were very similar to those seen in cultures inoculated with aquifer sediments previously exposed to sulfolane (4.6 to 6.4 mg/L/h). These results were not expected, given the much longer lag times shown by uncontaminated soil cultures (200 to 269 versus contaminated soil cultures (16 to 29 h). Also in favor of the hypothesis that degradation rates would be lower in cultures made up of pristine soils is the possibility that acclimation to the compound had occurred in contaminated sediments. The pristine soils did not have a chance to develop a sulfolane-degrading population before the time of inoculation, whereas presumably such a population was already present in contaminated soils, at least one of which had been exposed to the compound for more than 30 y. These results suggest that once a sulfolane-degrading microbial population had developed in a given culture, removal of the compound proceeded efficiently, given the

Table 3.2. The lag time and zero-order rate of sulfolane removal from cultures refed with sulfolane after the compound had initially been removed. The increase in biodegradation rate after refeeding was calculated relative to the average initial biodegradation rate (Table 3.1). Average values for lag times and degradation rates are given; the individual values are shown in brackets.

soil or sediment inoculum in culture	incubation temperature	lag time (h)	zero-order rate of degradation (mg/L/h)	rate increase (x-fold)
site 1 contaminated	8°C	2	1.8 (1.4-2.1)	1.4
site 2 contaminated	8°C	<1	15 (14-15)	6.3
site 3 contaminated	8°C	<1	5.3 (5.1-5.4)	2.8
site 1 contaminated	28°C	<1	18 (17-19)	2.8
site 2 contaminated	28°C	<1	44 (43-45)	9.6
site 3 contaminated	28°C	<1	19 (17-21)	3.9

available nutrients and other conditions which governed the biodegradation rate. The amount of sediment material used to inoculate each sample also did not affect the biodegradation rate or lag time; because of a shortage of sample, the site 3 cultures received only 35 g of aquifer sediment, rather than 50 g. The site 3 cultures showed the longest lag time only when incubated at 28°C and had faster degradation rates than cultures inoculated with site 1 materials at 8°C and site 2 materials at 28°C (Table 3.1). It is possible that in the case of the uncontaminated soil cultures, the large bacterial population present in the nutrient-rich greenhouse and humus-rich soils allowed a sulfolane-degrading bacterial population to develop more quickly than the relatively low bacterial population in the uncontaminated aquifer sediment. Fewer cultureable cells and a smaller variety of colony morphologies were observed in the uncontaminated aquifer sediment than in the greenhouse and humus-rich soils when the three were examined on LB agar and PAT medium (Section 2.10).

Fedorak and Coy (1996) studied sulfolane biodegradation in shake-flask experiments using samples from the site 1 sour gas treatment plant. In the absence of N and P supplementation, sulfolane biodegradation rates of 0.03 mg/L/h and 0.04 mg/L/h were observed (Fedorak and Coy 1996); these values are approximately 10-fold lower than those demonstrated in the current shake-flask experiments. In the earlier study, only groundwater was used as the source of microorganisms, whereas in this study sediment, which is a rich source of microorganisms (Holm et al. 1992), was also included in the shake-flask cultures. The marked difference in rates is likely due to the presence of sediment in the present study.

The rate constants determined in the shake-flask (slurry) cultures (Tables 3.1 and 3.2) are unlikely to be useful for predicting biodegradation of sulfolane at the three sampling sites. These cultures were supplemented with nutrients such as N and P and aerated through vigorous shaking, which also increased the mixing of sulfolane and nutrients with the sediment inoculum. Such conditions are likely to have promoted substantially higher biodegradation rates than would be observed in sulfolane-contaminated aquifers. These experiments have demonstrated that sulfolane-degrading populations exist in aquifer sediments previously exposed to sulfolane, and that such populations can develop over a period of 1 to 2 wk in a vigorously aerated, nutrient amended pristine soil or sediment. The development of such a population under these highly enriched conditions suggests that over time, a sulfolane-degrading population could develop under field conditions; the presence of sulfolane-degrading bacterial populations in contaminated sediments supports this hypothesis.

In the aquifer, the sediment would be compacted. Fu et al. (1996) used electrolytic respirometry to compare O_2 uptake rates during phenol degradation in slurry and compacted soil tube reactors. It was hypothesized that the tube reactor would more closely represent field conditions, since it was dependent on diffusion of nutrients without mixing. When different reactors were challenged with 3.75 mg of phenol, the O_2 uptake rates were 10-fold greater in a shake-flask slurry reactor than in the compacted soil tube reactor, and the total O_2 uptake by the slurry reactor was double that of the other reactor, indicating greater phenol degradation. Sulfolane degradation is similarly predicted to be substantially slower in compacted aquifer sediments than in the shake-flask slurry cultures examined here.

3.2.2 Anaerobic microcosms

3.2.2.1 Acetate-containing microcosms

Some anaerobic microcosms inoculated with sediments from the three sites investigated were given acetate as a C source and used to detect active microbial populations utilizing the various terminal electron acceptors tested: NO_3^-, Mn(IV), Fe(III), $SO_4^{=}$ and CO_2 (Section 2.9.1.2). Acetate is used by a wide variety of anaerobic microbial populations (Christensen, 1994). The possibility of inhibition by compounds or conditions present in any of the aquifer sediments was also tested. For each of the five anaerobic conditions used, a positive control microbial culture able to utilize acetate under those conditions was inoculated into microcosms containing medium with or without aquifer sediment. The organisms used to inoculate these positive control cultures were: *Pseudomonas* sp. W1 for NO_3^--reducing conditions, *Geobacter metallireducens* for Mn(IV)- and Fe(III)-reducing conditions, *Desulfobacter hydrogenophilus* for $SO_4^{=}$-reducing conditions and anaerobic sewage sludge for methanogenic conditions. Each microcosm was supplemented with approximately 5 mM acetate, and refed as required.

In all of the positive control microcosms, samples inoculated with acetate-utilizing organisms and sediment showed more activity than samples inoculated only with acetate-utilizing microbes (Table 3.3). In the NO_3^--reducing microcosms, the *Pseudomonas* sp. W1 did not utilize acetate; only those samples receiving sediment showed significant signs of NO_3^- reduction or acetate removal. This result was not expected, however acetate utilization occurred in sediment-inoculated samples, therefore it was unlikely that NO_3^--reducing microorganisms were inhibited by the

Table 3.3. Microbial utilization of acetate and the consequent change in terminal electron acceptor under anaerobic conditions in microcosms inoculated with positive control organisms (see text) with or without aquifer sediment. Microcosms were incubated at 28°C for the NO_3^--reducing microcosms and 37°C for the others. Changes in the following terminal electron acceptors (TEAs) were monitored: decreased NO_3^- and increased Mn(II), Fe(II), sulfide and methane. Microcosms were incubated up to 240 d.

TEA	soil added to micro-cosm	site 1		site 2		site 3	
		acetate used (mmol)	change in TEA (mmol)	acetate used (mmol)	change in TEA (mmol)	acetate used (mmol)	change in TEA (mmol)
NO_3^-	+	0.6	-1.3	2.4	-2.5	0.8	-0.9
NO_3^-	-	0.1	-0.3	0	-0.2	0.1	0
Mn(IV)	+	0.6	+0.5	0.7	+1.1	0.5	+0.9
Mn(IV)	-	0.4	+0.2	0	+1.9	0.03	+1.1
Fe(III)	+	0.6	+2.1	0.6	+5.9	0.2	+3.0
Fe(III)	-	0.3	+0.2	0	+2.1	0.01	+14
$SO_4^=$	+	0.7	+[a]	2.7	+[a]	0.7	+0.2
$SO_4^=$	-	0.7	+[a]	0.7	+[a]	0.7	+0.3
CO_2	+	0.7	+11 %[b]	0.6	ND[c]	0.4	ND[c]
CO_2	-	0.7	+13 %[b]	0	ND[c]	0.1	ND[c]

[a] The presence of sulfide was detected in sediment slurry samples from this culture, using a modified version of the methylene blue assay (Standard Methods, 1989).

[b] % methane detected in the headspace of microcosms.

[c] ND not detected

conditions present in these microcosms.

G. metallireducens showed lower levels of acetate oxidation coupled to Mn(IV) and Fe(III) reduction in the absence of added sediment than in its presence, suggesting that the incubation conditions were not optimal for this organism. The anaerobic sewage sludge produced methane only in one set of samples (Table 3.3). The lack of methane production in the other microcosms may have been due to the inoculum being older and less active at the time the microcosms were inoculated or to inhibition by the presence of contaminants in the sediment.

Acetate was depleted in all the microcosms that were inoculated with aquifer sediment and incubated under CO_2-reducing conditions; this was likely due to the use of terminal electron acceptors such as $SO_4^=$ that were present in the sediment. The presence of $SO_4^=$ and $SO_4^=$-reducing bacteria in aquifer sediments was demonstrated in NO_3^--reducing microcosms inoculated with site 2 sediment; after all the NO_3^- had been removed from these microcosms, acetate utilization continued and a black precipitate formed in the sediment. Analysis of this blackened sediment using the methylene blue assay (Standard Methods, 1989) demonstrated the presence of sulfide. *D. hydrogenophilus* was able to stimulate acetate removal coupled to $SO_4^=$ reduction in all cases (Table 3.3).

Although these data demonstrated that a number of the positive control organisms were insufficient to stimulate acetate oxidation coupled to reduction of the appropriate terminal electron acceptor, acetate utilization occurred in the sediment-inoculated positive controls. Therefore sediment in the positive control samples did not interfere with microbial utilization of acetate, and the sediment was unlikely to contain compounds inhibitory to microbial activity under anaerobic conditions.

The only exception to this rule was in the cases of methanogenic and $SO_4^=$-reducing microcosms. The methanogenic population present in site 1 aquifer sediments did not appear to interfere with $SO_4^=$ reduction by the *D. hydrogenophilus* inoculum. In contrast, $SO_4^=$-reducing populations were observed at sites 2 and 3, whereas no methanogenesis was detected. This result was not entirely surprising since $SO_4^=$-reducing bacteria can outcompete methanogens for acetate (Christensen et al., 1994; Grbić-Galić, 1990). Sulfate was detected in sediment samples taken from sites 2 and 3 (data not shown), which would likely have allowed the development of $SO_4^=$-reducing bacteria, thereby repressing the methanogens in these sediments.

In all acetate-fed, sediment-inoculated microcosms incubated under NO_3^--reducing conditions, NO_3^- concentrations decreased concomitantly with acetate consumption (Table 3.4). Populations were active at both 8°C and at 28°C, and were able to remove similar amounts of acetate at similar rates (data not shown). This suggested that NO_3^--reducing populations were present in all contaminated aquifer sediments, and that incubation at a higher temperature did not affect their activity.

Mn(IV)-reducing populations also were present at all three sites (Table 3.4). The production of Mn(II) coupled with acetate consumption was not strongly affected by incubation at 28°C rather than at 8°C. Site 3 sediment contained approximately 990 mg/L Mn(II) at the time of sampling. The site 3 microcosms in particular were predicted to have a Mn(IV)-reducing population, due to this observation. Lovley (1991) stated that the presence of reduced Fe or Mn may indicate the presence of an active Fe- or Mn-reducing population at a site. Microcosms inoculated with site 3 aquifer sediment demonstrated greater acetate consumption coupled to Mn(II) production than sediments from sites 1 and 2, which had less detectable Mn(II) at time zero (95 mg/L and 340 mg/L, respectively).

Fe(III)-reducing populations were observed at all three sites (Table 3.4). Incubation at 28°C appeared to stimulate acetate utilization coupled to Fe(III) reduction in microcosms from site 1, however those from sites 2 and 3 demonstrated similar activity at both incubation temperatures.

Sulfate-reducing activity was not detected at site 1, however microcosms from site 2 contained a very active $SO_4^{=}$-reducing population, and the microbial population in site 3 microcosms was also able to couple acetate oxidation with $SO_4^{=}$ reduction (Table 3.4). The NO_3^--reducing microcosms from site 2 demonstrated $SO_4^{=}$-reducing activity; after all the NO_3^- had been removed the residual acetate was slowly removed, and a black precipitate formed in the microcosms. Before sampling, this precipitate appeared as black flecks throughout the sediment in the microcosms, suggesting that it was produced at several positions within each microcosm. Sediment slurry samples contained sulfide, detected using the methylene blue assay (Standard Methods, 1989). No such precipitate formed in any of the sterile control samples, however this precipitate was observed in $SO_4^{=}$-reducing microcosms that had not been supplemented with NO_3^-. These data suggested that a well developed $SO_4^{=}$-reducing microbial community was present in aquifer sediments at site 2.

Table 3.4. Microbial utilization of acetate and the consequent change in terminal electron acceptor in anaerobic microcosms inoculated with sediment from sites 1, 2 or 3 and incubated for up to 240 d.

		site 1		site 2		site 3	
TEA	°C	acetate used (mmol)	change in TEA (mmol)	acetate used (mmol)	change in TEA (mmol)	acetate used (mmol)	change in TEA (mmol)
NO_3^-	8	0.5	-1.1	2.6	-2.4	0.7	-1.0
	28	0.7	-1.2	2.6	-2.3	0.8	-1.6
Mn(IV)	8	0.6	+0.5	0.7	+1.1	0.7	+1.0
	28	0.7	+0.7	0.6	+1.0	0.6	+1.6
Fe(III)	8	0.7	+2.3	0.7	+3.2	0.7	+1.5
	28	0.6	+3.1	0.6	+3.1	0.6	+1.9
$SO_4^{=}$	8	0.03	NM[a]	2.0	+[c]	0.8	+0.3
	28	0.03	NM[a]	1.4	+[c]	0.8	+0.5
CO_2	8	0.4	+0.40 %[b]	0.3	ND[d]	0.2	ND[d]
	28	0.7	+11 %[b]	0.6	ND[d]	0.2	ND[d]

[a] NM not measured

[b] % methane detected in the headspace of microcosms.

[c] The presence of sulfide was detected in sediment slurry samples from this culture, using a modified version of the methylene blue assay (Standard Methods, 1989), however sulfide was not quantified by this method.

[d] ND not detected

Addition of site 2 aquifer sediment to methanogenic contributed approximately 200 mg/L $SO_4^=$. Sulfate-reducing bacteria were predicted to be less active in samples where metal-reducing populations were present (Lovley, 1991), however if the total oxidized N, Fe and Mn species were depleted, the development of a $SO_4^=$-reducing bacterial population would be expected, particularly where $SO_4^=$ was available, as at site 2.

Methanogenesis was observed only in microcosms containing aquifer sediments from site 1 (Table 3.4). Some acetate was utilized in the site 2 and 3 microcosms, without methane production. It is likely that terminal electron acceptors other than CO_2 were used for acetate oxidation in these cases. Sulfate (200 mg/L in microcosms) and Fe(III) (210 ± 25 mg/L in microcosms) were detected in site 2 aquifer sediments; either could serve as terminal electron acceptors under methanogenic conditions. It has been previously observed that $SO_4^=$ or Fe(III) reduction will take precedence over methanogenesis (Grbić-Galić, 1990; Lovley, 1991), because iron reduction and $SO_4^=$ reduction are both more thermodynamically favorable (Table 1.3, Christensen et al. 1994). The competition offered by the presence of a $SO_4^=$- or Fe(III)-reducing population can prevent a methanogenic population from developing. At site 2 $SO_4^=$ is readily available, therefore methanogenic bacteria likely could not compete well for acetate in these microcosms. No black sulfide precipitate was observed in the site 2 CO_2-reducing microcosms, however much less acetate was utilized than in microcosms where the black precipitate was evident, and Fe(III) was also available. The data from this experiment supports the observation that the only detectable methanogenic population occurred in an aquifer sediment with no detectable $SO_4^=$-reducing population.

The results from the anaerobic microcosms containing acetate suggest the following.

1. It is unlikely that the aquifer sediments or incubation conditions were inhibitory to microbial activity under any of the five anaerobic redox conditions investigated in this experiment.
2. Nitrate-, Mn(IV)-, and Fe(III)-reducing populations were present in aquifer sediments at all three sites investigated.
3. Sulfate-reducing populations were present in aquifer sediments at sites 2 and 3.
4. Methanogenic populations were present in aquifer sediments at site 1.

These data were used to predict the potential for anaerobic sulfolane biodegradation in the contaminated aquifer sediments at each site.

3.2.2.2 Sulfolane-containing microcosms

3.2.2.2.1 Feasibility of sulfolane biodegradation under anaerobic conditions

Before developing an experimental method to determine whether anaerobic biodegradation of sulfolane occurred at any of the three sites surveyed for microbial activity against this compound, calculations were made to determine that anaerobic sulfolane biodegradation was thermodynamically feasible. As methanogenesis was felt to yield the least possible energy from sulfolane biodegradation, the Gibb's free energy change for sulfolane removal under methanogenic conditions was calculated.

The thermodynamic feasibility for sulfolane biodegradation under methanogenic conditions was predicted using the Gibb's free energy change. The free energy of formation for sulfolane is -2.59 x 10^8 J/kmol (Aspen Technology Inc.) The biodegradation of sulfolane under methanogenic conditions can be expressed by equation, based on the general formula given by Symons and Buswell (1933). Equations describing sulfolane biodegradation under the five anaerobic conditions tested, including methanogenic conditions, are shown in Table 3.5. The $\Delta G^{\circ\prime}$ for sulfolane degradation to CO_2, CH_4 and H_2S under methanogenic conditions is -233 kJ/mol sulfolane. This calculation was made by P.M. Fedorak (Dept. of Biological Sciences, University of Alberta, Edmonton, AB) using the free energy values given by Thauer et al. (1977). This indicates that sulfolane biodegradation is thermodynamically favorable under anaerobic conditions with all the terminal electron acceptors used in this research, methanogenesis being the least favorable of those tested. This activity was predicted to depend on active microbial populations able to utilize this compound.

3.2.2.2.2 Sulfolane-degrading bacterial populations under different anaerobic conditions

For each of the three sites investigated, five anaerobic conditions were tested: $NO_3^{\cdot}$-, Mn(IV)-, Fe(III)-, $SO_4^{=}$- and CO_2-reducing (methanogenic). Two viable and one sterile control microcosms inoculated with aquifer sediments from each of the three sites were incubated at 8°C and at 28°C; in all this represented 60 viable and 30 sterile control microcosms supplemented with sulfolane. Microcosms containing no added C source were inoculated with site 1 aquifer sediment and incubated under the five anaerobic conditions; these acted as controls to demonstrate that any observed changes in the terminal electron acceptors were coupled to the oxidation of the added C source; in this case, sulfolane. No changes in any of the terminal electron acceptors were observed in any of these control microcosms. No changes in sulfolane concentration or

Table 3.5. Stoichiometric reactions describing the oxidation of sulfolane in the presence of different terminal electron acceptors (TEAs). The Gibb's free energy change for 1 mol sulfolane (calculated based on each equation as it is shown) is given where it is available.

TEA	equation	ΔG° (kJ/ mol)
O_2	$C_4H_8O_2S + 6.5O_2 + H_2O \rightarrow 4HCO_3^- + 6H^+ + SO_4^=$	NC[a]
NO_3^-	$5C_4H_8O_2S + 18NO_3^- \rightarrow 5HS^- + 9N_2 + 20HCO_3^- + 4H_2O + 7H^+$	-1945
Mn(IV)	$C_4H_8O_2S + 10H_2O + 9Mn^{4+} \rightarrow S^= + 9Mn^{2+} + 4HCO_3^- + 24H^+$	NC[a]
Fe(III)	$C_4H_8O_2S + 10H_2O + 18Fe^{3+} \rightarrow S^= + 18Fe^{2+} + 4HCO_3^- + 24H^+$	NC[a]
$SO_4^=$	$4C_4H_8O_2S + 4H_2O + 9SO_4^= \rightarrow 13HS^- + 16HCO_3^- + 11H^+$	NC[a]
CO_2	$4C_4H_8O_2S + 6H_2O \rightarrow 7CO_2 + 9CH_4 + 4H_2S$	-233

[a] NC not calculated.

in the terminal electron acceptors were observed in any of the sterile control microcosms incubated at 8°C or at 28°C. The experimental design is explained in Section 2.9.1.1.

The anaerobic microcosms from sites 1, 2 and 3 were monitored for 390, 504 and 446 d, respectively. No sulfolane degradation was observed in any of the viable microcosms incubated at 28°C. This result is in contrast to the results observed for acetate utilization in microcosms with the same aquifer sediment inocula, where oxidation of the C source occurred at both temperatures.

Of the microcosms incubated at 8°C, only 4 of the 30 anaerobic microcosms supplemented with sulfolane showed any evidence of sulfolane biodegradation (Table 3.6). No anaerobic sulfolane degradation was observed in any microcosms containing sediment from site 2. Site 1 yielded the most active sediment, with evidence for sulfolane biodegradation under NO_3^-- and Mn(IV)-reducing conditions. There was also evidence that sulfolane was degraded under Mn(IV)-reducing conditions in one microcosm that contained sediment from site 3. Loss of sulfolane was observed in one Fe(III)-reducing microcosm containing site 1 sediment, however this microcosm appeared to have very low levels of activity (Figure 3.3), and the amount of Fe(II) production was not deemed adequate to demonstrate that Fe(III) reduction coupled to sulfolane oxidation had occurred in the microcosm.

Few sulfolane-degrading populations were detected, particularly in the presence of terminal electron acceptors that yielded less energy per molecule of substrate oxidized, such as Fe(III), $SO_4^=$ or CO_2. The acetate-supplemented microcosms inoculated with the same aquifer sediments demonstrated much more activity than the sulfolane-supplemented microcosms, particularly those inoculated with aquifer sediments from site 2 (Tables 3.3, 3.4, 3.6). It is unlikely that the presence of sulfolane in the groundwater at site 2 proved toxic to the microbial population. Although this site had the highest groundwater sulfolane concentration of the three investigated (310 mg/L, as opposed to 4 mg/L and 30 mg/L for sites 1 and 3, respectively), acetate utilization at the site readily occurred, therefore an active microbial population was demonstrated to exist at this site. Given the higher level of contamination in the groundwater at site 2, one would predict that more microbial activity against sulfolane, rather than less, would be likely. It is probable that the lack of activity against sulfolane in site 2 aquifer sediment is due to the inability of the appropriate bacterial population to develop. This inactivity cannot, however, be explained by the data obtained from the anaerobic microcosm experiments.

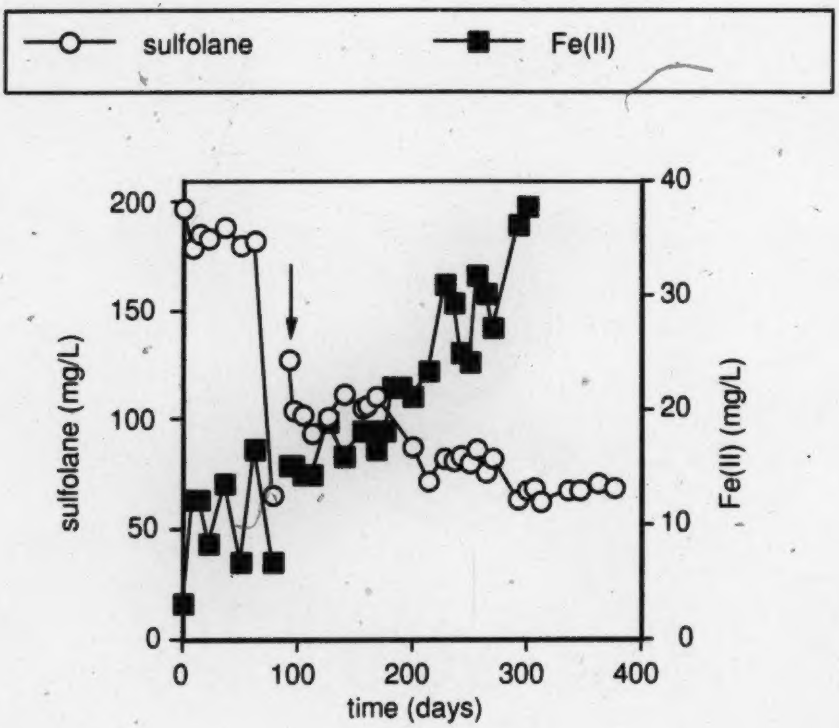


Figure 3.3. Loss of sulfolane and the production of Fe(II) in an anaerobic microcosm inoculated with site 1 sediment, amended with $Fe(OH)_3$, and incubated at 8°C. Total production of Fe(II) was low, and the activity of this microcosm against sulfolane decreased during the incubation. The arrow indicates the time at which the microcosm was refed with sulfolane and $Fe(OH)_3$.

Table 3.6. Summary of the lag times before biodegradation of sulfolane and the rate of sulfolane removal in anaerobic sulfolane-degrading microcosms in the presence of different terminal electron acceptors (TEAs). Replicate microcosms incubated at 8°C were designated A and B; transfer microcosms were designated A or B depending on the replicate microcosm from which they were inoculated. The number of times each microcosm was fed is indicated in the sulfolane supplementation column.

site	TEA	culture designation	sulfolane supplementation	lag time (d)	degradation rate (mg/L/d)
1	NO_3^-	replicate B	#1 (190 mg/L)	35	5
		replicate B	#2 (210 mg/L)	<3	5
		replicate B	#3 (220 mg/L)	<3	5
		transfer B[a]	#1 (250 mg/L)	103	1.5
1	Mn(IV)	replicate A	#1 (190 mg/L)	160	3
		replicate B	#1 (180 mg/L)	34	5
		replicate B	#2 (160 mg/L)	<3	1
		transfer A[a]	#1 (210 mg/L)	<7	0.4
		transfer B[b]	#2 (230 mg/L)	157	0.2
3	Mn(IV)	replicate A	#1 (200 mg/L)	168	1
		replicate A	#2 (220 mg/L)	<3	3
		replicate A	#3 (190 mg/L)	<3	5
		transfer A[a]	#1 (200 mg/L)	70	0.4

[a] 20 mL of active microcosm slurry was transferred to 55 mL medium containing 10 mL MnO_2 slurry or 65 mL medium containing NO_3^-

[b] 10 mL of active microcosm slurry was transferred to 55 mL medium containing 10 mL MnO_2 slurry

The lack of development of sulfolane-degrading populations under various redox conditions at the three sites could be due at least in part to competition for nutrients from bacterial species capable of growing on more easily degraded C sources. The presence of sulfolane in the groundwater at detectable concentrations at all three sites, over a period of years, suggests that should a sulfolane-degrading population have been likely to develop in these aquifers, it would have been detectable. The acetate utilization experiments demonstrated that bacterial populations able to utilize four of the five terminal electron acceptors tested were present in each aquifer sediment sample. A much broader portion of the microbial population in the aquifer sediments was expected to be able to utilize acetate as a C source than sulfolane. Sulfolane is a compound in which the available C is in a ring structure, therefore less accessible to bacterial utilization than in a small, straight chain compound such as acetate. The thermodynamic considerations demonstrated in Table 3.5 support the hypothesis that the inability to demonstrate sulfolane degradation under any conditions at site 2, and under Fe(III)-reducing, $SO_4^{=}$-reducing and methanogenic conditions at sites 1 and 3, was due to the absence of the appropriate active microbial communities, rather than to thermodynamically unfavorable reactions. However, these values for the Gibb's free energy change also suggest that should a substrate easier to degrade than sulfolane be available, it would be more important for microorganisms that could get less energy per molecule of substrate to utilize this compound that was easier to degrade, thereby saving energy, than it would be for organisms that could degrade sulfolane less expensively.

3.2.2.2.3 Sulfolane biodegradation under nitrate-reducing conditions

Loss of sulfolane was observed in the one of the microcosms containing contaminated sediment from site 1 incubated in NO_3^--supplemented medium at 8°C (Figure 3.4A). When the sulfolane concentration in this microcosm had decreased from 190 mg/L to undetectable levels after 159 d of incubation, the microcosm was refed with NO_3^- and sulfolane (Figure 3.4A), and 20 mL of the slurry from the microcosm was transferred to fresh medium (Figure 3.4B). The original NO_3^--supplemented microcosm removed the second amount of sulfolane from 210 mg/L to 21 mg/L, without a significant lag, over a period of 50 d, after which the microcosm was refed sulfolane without additional NO_3^-. The sulfolane concentration in this microcosm again decreased with no significant lag period, from a starting concentration of 220 mg/L. However, once a concentration of approximately 40 mg/L sulfolane was reached, no further degradation was observed (Figure 3.4A). This may have been due to the buildup of NO_2^- and N_2O, which are toxic intermediates or products of NO_3^- reduction (Schulthess et al., 1995). A large excess of NO_3^- was present in this microcosm; therefore NO_3^- reduction would not necessarily be driven to N_2 due to limited availability of an acceptable terminal electron acceptor, thus encouraging the buildup of these toxic intermediates. This hypothesis is supported by the detection of N_2O in the culture. Throughout the 375-d incubation, the mean sulfolane concentration in the corresponding sterile control was 190 ± 8 mg/L (data not shown). The lag times and zero-order rate constants for sulfolane loss from the NO_3^--reducing microcosm are summarized in Table 3.6.

During the initial loss of sulfolane, NO_3^- concentrations decreased from 1150 to 1060 mg/L (Figure 3.4A). After the culture had been refed sulfolane and NO_3^-, NO_3^- concentrations decreased from 2150 to 1950 mg/L. The third feeding of sulfolane led to NO_3^- concentrations decreasing from 1950 to 1780 mg/L. The mean NO_3^- concentration in the corresponding sterile control over the incubation time was 1420 ± 45 mg/L.

Overall, 0.31 mmol of sulfolane and 0.46 mmol of NO_3^- were consumed in this microcosm over the 375-d incubation. The complete mineralization of sulfolane under NO_3^--reducing conditions is predicted to require 18 mol of NO_3^- per 5 mol of sulfolane, assuming that the NO_3^- is reduced to N_2 (Table 3.5). Based on this prediction, the microcosm utilized only 42% of the 1.11 mmol NO_3^- required to mineralize the 0.31 mmol of sulfolane that were consumed. This suggests that sulfolane may not have been completely mineralized in this microcosm.

The NO_3^--supplemented transfer microcosm showed an initial lag period of 103 d followed by a decrease in sulfolane of approximately 1.5 mg/L/d, with concentrations dropping from an initial 250 mg/L to 80 mg/L (Figure 3.4B). The NO_3^- concentration in this transfer culture decreased from 1340 mg/L to 1080 mg/L during the time that the sulfolane was removed. Overall, 0.11 mmol of sulfolane and 0.31 mmol of NO_3^- were consumed in this microcosm, therefore 78% of the predicted 0.4 mmol of NO_3^- were consumed.

An ample supply of NO_3^- was present in all microcosms, therefore it is unlikely that the terminal electron acceptor was limiting. GC analyses of the headspace gases from the refed microcosm and the transfer culture showed the presence of N_2O, which is an intermediate or a product of NO_3^- reduction (Firestone et al., 1980; Davidson, 1991). N_2O was not detected in the corresponding sterile control. This provided evidence that NO_3^- reduction occurred in both the microcosms under discussion, and that it was likely

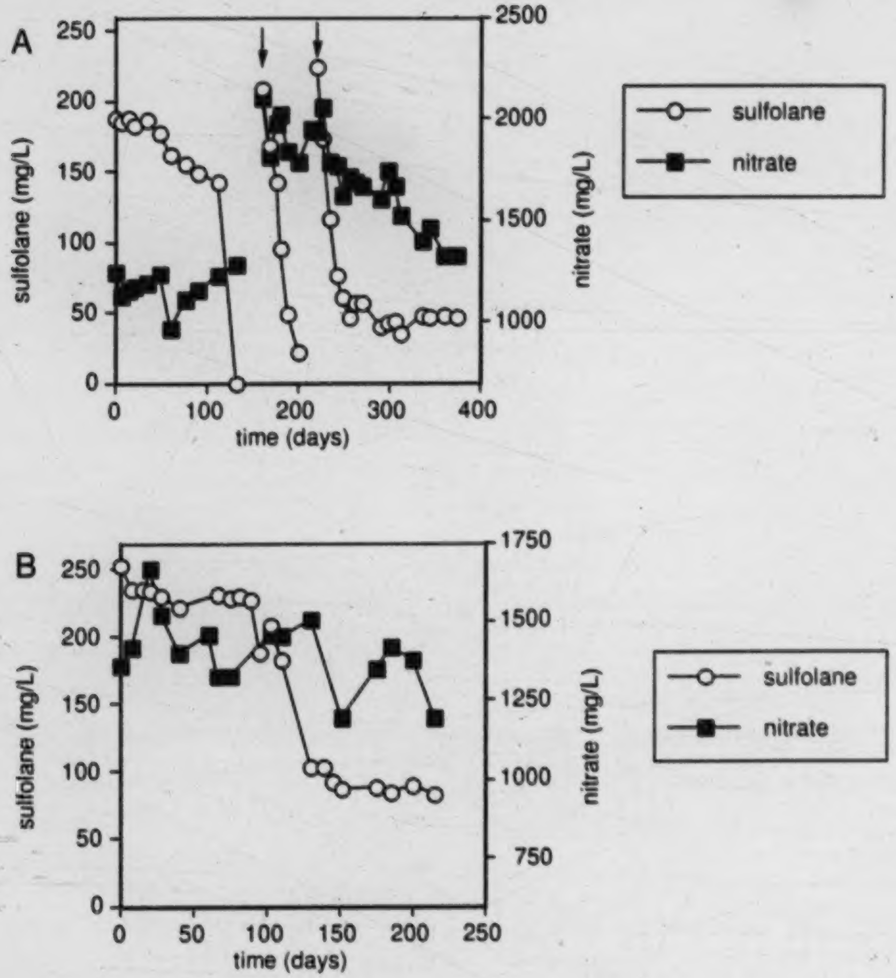


Figure 3.4. Loss of sulfolane and the corresponding loss of NO_3^- from anaerobic microcosms inoculated with site 1 aquifer sediment, amended with NO_3^-, and incubated at 8°C. A. Replicate A microcosm. The first arrow indicates the time at which the microcosm was reamended with sulfolane and NO_3^-. The second arrow indicates reamendment with sulfolane only. B. Transfer A microcosm amended with NO_3^- and sulfolane.

coupled to sulfolane degradation. The production of N_2 by NO_3^- reduction and CO_2 from sulfolane mineralization were not measurable in these microcosms; the headspace gas initially contained 30% CO_2 and 70% N_2, preventing the accurate measurement of production of either of these gases.

The presence of N_2O, while it supports NO_3^- reduction, also supports the hypothesis that sulfolane was transformed, rather than mineralized, in these microcosms. Calculations suggested that should NO_3^- reduction have gone completely to N_2, only 42% and 78% of the NO_3^- consumption predicted for the original and transfer microcosms, respectively, actually occurred. Should NO_3^- have only been reduced to N_2O rather than N_2, even less of the required NO_3^- reduction for sulfolane mineralization actually took place. Other investigators working with sediment-containing microcosms have found better agreement between the expected and observed amounts of NO_3^- consumed; for example, Häggblom et al. (1990) observed that 90% of the expected amount of NO_3^- was used during anaerobic biodegradation of *p*-cresol by microcosms that contained pond sediment. This also suggests that sulfolane metabolism may not have been complete in the site 1 microcosms. Twice, 3-mL samples of sediment slurry were removed from the NO_3^--reducing microcosms and extracted with diethyl ether and water in order to discover some S-containing metabolite of sulfolane transformation in these microcosms, however no compounds other than sulfolane were detected. Further attempts to detect sulfolane metabolites were not made due to the small volume of the active cultures. The goal of this work was to determine whether sulfolane could be biodegraded under NO_3^--reducing conditions by microbial communities from contaminated aquifers at sour gas plants; this activity was demonstrated. Determining a mass balance and identifying the microbial products of this biodegradation will require further studies. In order to carry out such studies, a fresh aquifer sediment sample and many more replicate microcosms would be required; very little could be done with a single active microcosm which contained only 75 mL medium and 50 g sediment before any samples had been removed.

3.2.2.2.4 Sulfolane biodegradation under Mn(IV)-reducing conditions

Loss of sulfolane under Mn(IV)-reducing conditions was observed in three microcosms incubated at 8°C (Table 3.6). The shortest lag time (34 d) was observed in the replicate B microcosm containing sediment from site 1, where the sulfolane concentration decreased from 180 to 20 mg/L by 91 d (Figure 3.5B). After refeeding at 92 d, this microcosm showed an immediate decrease in sulfolane; over a period of 207 d, the sulfolane concentration decreased from 160 mg/L to 5 mg/L, at a mean rate of 1 mg/L/d. This rate was substantially lower than rate of removal of the initial sulfolane dose.

In this microcosm, the Mn(II) concentrations increased from 90 to 190 mg/L during the initial loss of 162 mg/L of sulfolane, then increased further to 390 mg/L (Figure 3.5B) during removal of the second dose of sulfolane. Over the course of the experiment, 0.18 mmol of sulfolane was consumed and 0.26 mmol of Mn(II) was produced. The color of solids in the Mn(IV)-reducing microcosms also indicated Mn(IV) reduction; as described by Lovley and Phillips (1988), the dark brown precipitate of MnO_2 added to the microcosms was converted to a greyish-white precipitate, which is consistent with the formation of the reduced product $MnCO_3$.

The replicate A microcosm containing sediment from site 1 degraded sulfolane under Mn(IV)-reducing conditions after a lag time of 160 days (Table 3.6). The sulfolane concentration decreased from 190 mg/L to undetectable levels (Figure 3.5A) over the next 54 d of incubation, giving a mean degradation rate of 3 mg/L/d. After refeeding, there was only a slight decrease in sulfolane concentration (15 mg/L), and no measurable increase in Mn(II) over the next 155 d of incubation. Over the 375-d incubation, the microbial community in this microcosm consumed 0.13 mmol of sulfolane and produced 0.18 mmol of Mn(II).

The transfer microcosms containing sediment slurry from site 1 that was able to couple sulfolane degradation with Mn(IV) reduction were not able to remove more than 25% of the sulfolane present, even after long incubation times (Figures 3.5D, E). The replicate B microcosm was transferred before the replicate A microcosm; due to the long lag time (103 d) demonstrated by this first transfer microcosm, twice the volume of active sediment slurry was transferred for the replicate A microcosm. This larger volume may have aided in decreasing the lag time before sulfolane biodegradation commenced in the replicate A microcosm (<7 d rather than 103 d), however in the end the transfer A microcosm was not more active in removing sulfolane than the transfer B microcosm. As well as the low degradation rates observed in the transfer microcosms, the rate of sulfolane removal from the replicate B microcosm slowed 5-fold after the first feeding (Table 3.6), therefore it is possible that Mn(IV) reduction coupled to sulfolane oxidation was not important in the site 1 aquifer sediments.

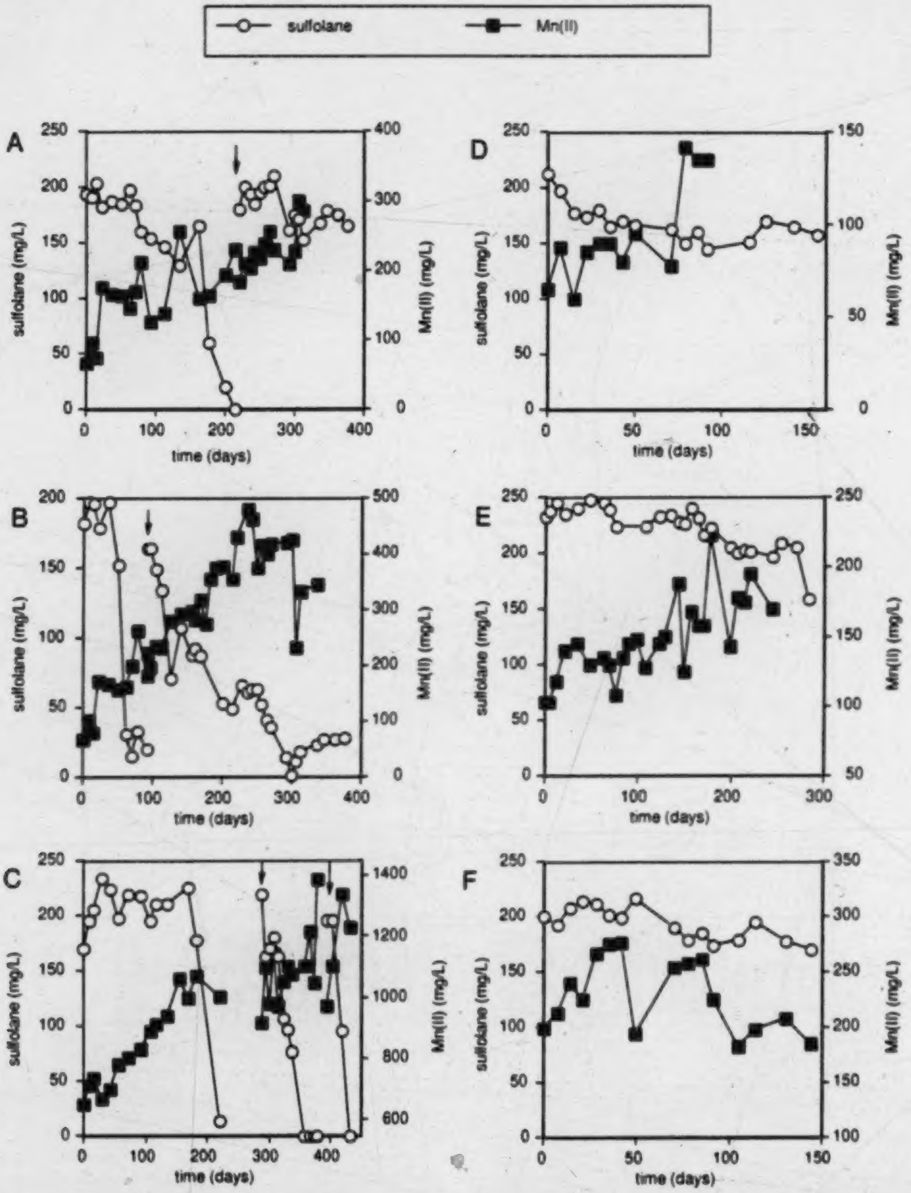


Figure 3.5. Loss of sulfolane and the corresponding increase in Mn(II) in anaerobic microcosms inoculated with site 1 or site 3 aquifer sediment, amended with MnO_2, and incubated at 8°C. A. Site 1 replicate A microcosm. B. Site 1 replicate B microcosm. C. Site 3 replicate A microcosm. D. Site 1 transfer A microcosm. E. Site 1 transfer B microcosm. F. Site 3 transfer A microcosm. Arrows indicate times at which microcosms were reamended with sulfolane and MnO_2.

The sulfolane concentration in the sterile control containing sediment from site 1 had a mean concentration of 180 ± 8 mg/L and a mean Mn(II) concentration of 110 ± 9 mg/L over the 375-d incubation period.

Mineralization of sulfolane under Mn(IV)-reducing conditions can be predicted to occur by the equation described in Table 3.5; 1 mol sulfolane would therefore require 9 mol MnO_2 for mineralization. For sulfolane mineralization in the site 1 microcosms, according to the equation in Table 3.5 the amounts of Mn(II) expected would be 1.15 and 1.57 mmol in replicates A and B, respectively. The measured formation in Mn(II) was 16% of the expected amounts in both microcosms. Given the low levels of sulfolane removal and Mn(II) production in the transfer microcosms (Figures 3.5D, E), it was not possible to compare the amount of Mn(II) formation and sulfolane removal in these microcosms.

The Mn(II) produced in the initial replicate microcosms may have become involved in the formation of compounds from which it was not extractable for detection, which would result in an underestimate of Mn(II) production in the microcosms, however this hypothesis is unlikely to explain the very low levels of Mn(II) that were detected. It is likely that sulfolane was not mineralized in these microcosms; with only 16% of the predicted Mn(II) production observed, one would expect that transformation of the compound, rather than mineralization, had occurred.

One site 3 microcosm showed evidence of sulfolane biodegradation under Mn(IV)-reducing conditions (Table 3.6, Figure 3.5C). After a lag period of 168 d, this microcosm removed 200 mg/L of sulfolane at 1 mg/L/d. After 268 d of incubation, this microcosm was refed and a portion was transferred to fresh medium. Sulfolane removal in the refed microcosm began without a lag period, decreasing from 220 mg/L to an undetectable level over a 70 d at 3 mg/L/d. This was unlike the site 1 active microcosms in that the sulfolane removal rate increased after the first feeding rather than decreasing. This increase in rate may have been due to the presence of a more active Mn(IV)-reducing population at site 3. High levels of Mn(II) were detected at site 3 before the microcosms were inoculated (Section 3.2.2.1), suggesting that a Mn(IV)-reducing population may have already been present at this site. The microcosm was refed a second time at 392 d of incubation. All of this substrate was removed after 39 d of incubation. The mean sulfolane concentration in the corresponding sterile control was 210 ± 8 mg/L over the 431-d incubation period. As with the transfer microcosms from site 1 replicate microcosms, the transfer microcosm from the active site 3 microcosm showed little ability to degrade sulfolane or reduce Mn(IV) (Figure 3.5F).

Mn(II) concentrations in the microcosm containing sediment from site 3 increased from 650 to 1000 mg/L during the removal of the initial dose of sulfolane. After the first refeeding, it increased to 1140 mg/L, and it increased further after the second refeeding to 1230 mg/L. The mean Mn(II) concentration in the corresponding sterile control was 920 ± 82 mg/L over the 431-d incubation period. The microbial community in this microcosm containing sediment from site 3 consumed 0.30 mmol of sulfolane and produced 0.70 mmol of Mn(II) over a period of 431 d. From the equation described in Table 3.5, the amount of Mn(II) production predicted to occur for mineralization of 0.30 mmol of sulfolane was 2.70 mmol, therefore only 26% of the predicted Mn(II) was produced. Incomplete metabolism of sulfolane may have left organic products in the medium, thereby requiring less MnO_2 to be reduced to Mn(II) than given by the equation, which presumes the complete mineralization of sulfolane. Thus, in all Mn(IV)-reducing microcosms that were able to utilize sulfolane, it was likely that this removal of sulfolane from the microcosm medium did not result in mineralization of the compound.

Microcosms from sites 1, 2, and 3 contained 85, 350 and 650 mg/L Mn(II) respectively, before Mn(IV) was added. These values differed from those found at time zero in the acetate-supplemented microcosms (Section 3.2.2.1), which were 95, 340, and 990 mg/L for the same sites, respectively. These differences were probably due to variability in the sample materials. Some variability was noticed between different replicate samples, in spite of the sediment being mixed before inoculation of the microcosms, therefore differences between samples in different parts of the experiment were not surprising. Lovley (1991) stated that measuring the dissolved Mn(II) in interstitial waters of sediments can provide a useful indication of the presence of Mn(IV)-reducing bacterial populations. Similarly, Gounot (1994) wrote that insoluble Mn(II) in the form of Mn_3O_4, $MnCO_3$ or adsorbed Mn(II) may constitute a significant portion of the Mn(II) generated from Mn(IV) reduction. My acid extraction protocol was likely to have allowed the detection of soluble Mn(II) from groundwater or microcosm medium and acid-soluble forms of insoluble Mn(II) in the sediments, therefore the presence of Mn(II) in the time zero samples suggests that Mn(IV)-reducing activities may have been occurring *in situ* at the sampling locations of the contaminated gas plant sites. The data for acetate utilization under Mn(IV)-reducing conditions supports the prediction that active populations were present at the 3 sites. Site 3 in particular appeared to have a large amount of Mn(II) present, and of the microcosms that were able to utilize sulfolane while reducing Mn(IV), the site 3 microcosm was most active (Table 3.6, Figure 3.5C).

The data collected from the microcosm studies suggest that sulfolane degradation via Mn(IV) reduction did not occur *in situ*. Only three of the twelve MnO_2-containing microcosms established with the contaminated sediments were able to degrade sulfolane, whereas all the microcosms supplemented with acetate showed Mn(IV)-reducing activity. Of the three active microcosms, two required lag times ≥ 160 d before sulfolane degradation commenced. Utilization of acetate commenced after lag times ranging from < 7 d to 14 d in microcosms inoculated with aquifer sediments from all three sites. One would expect shorter lag times before sulfolane degradation commenced if it were occurring coupled to Mn(IV) reduction *in situ*.

3.2.2.3 Sulfolane-containing anaerobic sewage sludge microcosms

Fresh anaerobic sewage sludge was obtained in order to determine whether an active methanogenic population was able to utilize sulfolane as a C source (Section 2.9.1.3). Sewage sludge microcosms were fed 50, 100, or 200 mg/L sulfolane. Positive control microcosms were supplemented with 1.25, 2.5 or 5 mM acetate as a C source. Acetate was removed from all the microcosms inoculated with anaerobic sewage sludge within 7 d of inoculation. Sulfolane was not removed from any of the microcosms over an incubation period of 90 d. It was concluded from this experiment that the methanogenic consortium present in a municipal waste water treatment plant was not capable of developing the ability to degrade sulfolane. Bridié et al. (1979), Chou and Swatloski (1983) and McLeod et al. (1992) found that activated sludge from various sewage treatment plants was initially unable to degrade sulfolane under aerobic conditions. However, Chou and Swatloski (1983) demonstrated that after an acclimation period of a few days, a population of sulfolane-degrading microorganisms developed in an aerated system. None of these authors investigated the possibility of anaerobic sulfolane degradation by activated sludge. It is possible that in the presence of the various C sources available in sewage sludge, no sulfolane-degrading population developed. No sulfolane-degrading population appeared to have developed in the site 1 contaminated aquifer sediment either, although a methanogenic population was present in this material (Tables 3.3 to 3.5). It was possible that although the biodegradation of sulfolane is thermodynamically favorable under methanogenic conditions (Table 3.5), some characteristic of the methanogenic conditions that were used in these microcosms was able to interfere with sulfolane utilization, or to repress the development of a bacterial population active against this compound. It was also possible that methanogenic bacteria were unable to use sulfolane due to problems with uptake, a lack of the appropriate enzyme systems, or other physiological deficiencies with respect to this compound. Methanogens generally use acetate, H_2, and a few other simple compounds as electron donors, therefore it was not entirely unexpected that they were unable to utilize sulfolane as a C and energy source.

3.3 The effect of nutrient amendment and incubation conditions on sulfolane biodegradation potential

Part of this research was initially reported by Greene et al. (in press). That information is presented here with the full knowledge and consent of all the co-authors.

The initial experiments to screen three contaminated sites for sulfolane degradation potential demonstrated that biodegradation of sulfolane by the microbial populations of contaminated aquifer sediments from sour gas plant sites occurred under highly aerobic, nutrient-supplemented conditions (Section 3.2.1). Soil microorganisms not previously exposed to sulfolane were able to degrade the compound, although they typically showed a much longer lag time before biodegradation began than contaminated sediment populations. All the cultures described in previous experiments were grown in the presence of supplementary inorganic nutrients rather than in conditions more closely related to those found in the environment.

Several authors have reported methods for examining biodegradation under aquifer-like conditions (Section 1.11). The purpose of the population and nutrient screening project was to determine whether simple laboratory experiments could be used to predict the sulfolane biodegradation potential of more aquifer-like systems. Bioremediation processes are known to be stimulated by the addition of inorganic N and P. The experiments done during this project assessed the effects of N and P supplementation on the lag times before the onset of sulfolane and DIPA biodegradation and on the rates of biodegradation. The portion of the project reported in this thesis relates mainly to sulfolane biodegradation.

Site 2 from the initial experiments done to screen three contaminated sites for sulfolane degradation potential (Section 3.2.1) was selected for the nutrient amendment tests. At site 2, groundwater containing sulfolane and DIPA has migrated from the contaminating landfill sources, and some sulfolane has been detected in the surface waters in a wetland that is downgradient from these sources (Figure 2.1). The sulfolane concentrations in the wetland were very low, therefore it appeared that the wetland might contain a microbial community that can degrade this compound. Uncontaminated aquifer sediments taken upgradient from the landfill cells, contaminated aquifer sediments taken from two downgradient sites and materials from wetlands at the far end of each contaminant plume were used as inocula in the following experiments (Figure 2.1). The two wetlands areas are separated by a beaver dam. The east wetland sample contained more fresh plant material and rich, black sediment, while the west wetland sample consisted more of mud and peat-like material.

3.3.1 Shake-flask cultures

The composition of the liquid phases of the shake-flask cultures containing aquifer sediments differed from the initial survey cultures (Section 3.2.1); this is detailed in Section 2.7.2.1. In the present study, the liquid phase consisted of groundwater from the contaminated location, whereas in the previous study it was B+NP-low N medium (Section 3.2.1). The liquid phase portion of these shake-flasks contained trace nutrients present in groundwater that were not necessarily present in a mineral medium, as well as organic contaminants other than sulfolane and DIPA (Table 2.2). The sulfolane concentration present in the east and west plume cultures was 2- or 3-fold higher than in the previous cultures, which were all supplemented with 200 mg/L sulfolane.

3.3.1.1 Presence and biodegradation of unidentified compounds in the aquifer samples

Table 2.1 shows that a considerable proportion of the total organic C present in the east plume and west plume groundwaters was contributed by compounds other than sulfolane, as observed by GC analyses (Figure 3.6), however these compounds were not identified. The presence of organic compounds other than sulfolane could affect the biodegradation of this compound in two ways. First, sulfolane biodegradation would be deferred if easily oxidized substrates were present, the more recalcitrant sulfolane molecule being less likely to be biodegraded until these compounds were exhausted. The C in sulfolane is in a heterocyclic ring; straight chain compounds such as DIPA, a common co-contaminant of sulfolane, and some hydrocarbons would likely be more accessible to bacteria than this structure. Hydrocarbons > C_5 and olefins are soluble in sulfolane (Chou and Swatloski, 1983; Merck Index, 1968), therefore may be present in sulfolane-contaminated sediments at sour gas treatment facilities or in other sites where sulfolane is employed as a solvent in the oil and gas industry. Olefins, or alkenes, are more soluble in water than alkanes (Fessenden and Fessenden, 1986), therefore could easily be present in contaminated groundwaters at these sites. Second, the presence of a limited amount of N, P or other essential nutrients could be depleted by biodegradation

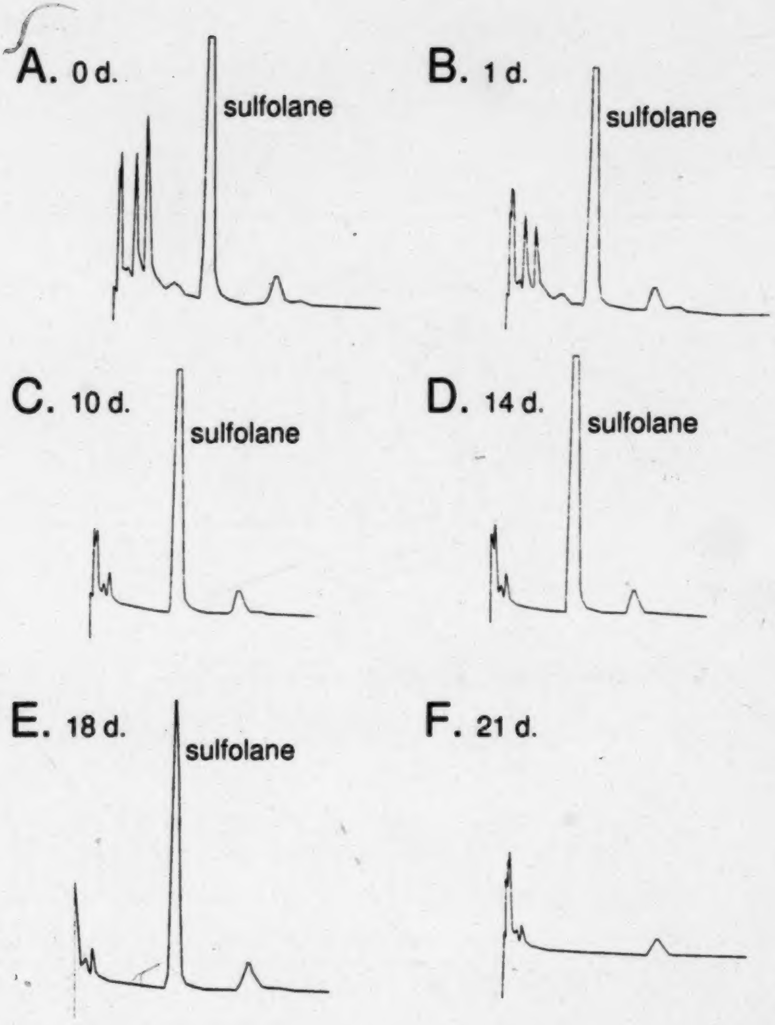


Figure 3.6. Direct aqueous injection GC analyses of samples from a shake-flask culture inoculated with aquifer sediment and groundwater form the site 2 east contaminated plume. The culture was supplied with $1.6P_r$. C-containing peaks other than sulfolane (co-contaminants) were evident in this sample. Samples were analyzed at intervals during incubation.

of organic compounds other than sulfolane, which would preclude subsequent biodegradation of sulfolane. These possibilities were not investigated.

3.3.1.2 Shake-flask cultures

Four cultures were incubated for each sampling site: an unsupplemented culture, one supplemented with 3.1 mg/L N and 79 mg/L P at time zero (this culture was amended to contain 31 mg/L N and 790 mg/L P after 14 d), one supplemented with 31 mg/L N and 790 mg/L P from time zero, and a sterile control. The culture initially supplemented with lower levels of N and P was used to determine whether low levels of N and P supplementation were sufficient to stimulate biodegradation of sulfolane.

The removal of sulfolane from shake-flask cultures by biodegradation is shown in Figure 3.7, and the lag times and biodegradation rates for the cultures are summarized in Table 3.7. The zero-order and first-order kinetic analyses for these cultures are shown in Table B2 in Appendix B.

Sulfolane biodegradation was observed in all the viable shake-flask cultures, and the compound was completely removed from all but one of the cultures; in the west plume unsupplemented shake-flask culture 48% of the initial dose of sulfolane remained after 150 d of incubation (Figure 3.7B). No further changes in this culture were observed after 224 d of incubation (data not shown). It is probable that other organic C compounds present in this culture were preferentially degraded, therefore some sulfolane remained when the culture became nutrient limited. In the aquifer sediment cultures that were not supplemented with N and P the lag times were generally quite long, ranging from 34 to 98 d (Table 3.7). A notable exception was the culture inoculated with materials from the west wetland region; the observed lag time was only 9 d in this culture.

The west wetland culture supplemented with a high level of P at time zero had the fastest degradation rate and the shortest lag time for sulfolane degradation of the west wetland cultures (Table 3.7). However, the west wetland unsupplemented culture had a shorter lag time before sulfolane degradation than the culture supplemented with a low level of N and P at time zero, which was surprising. These cultures were analyzed for the presence of NO_3^-, to demonstrate that the incorrect culture had not been amended with N and P, and it was found that the unsupplemented culture contained substantially less NO_3^- than the supplemented culture. This demonstrated that biodegradation in the west wetland cultures was not significantly improved by N and P supplementation, at least at low levels, unlike cultures inoculated with materials from the other sampling locations. The west wetland sample contained a considerable amount of

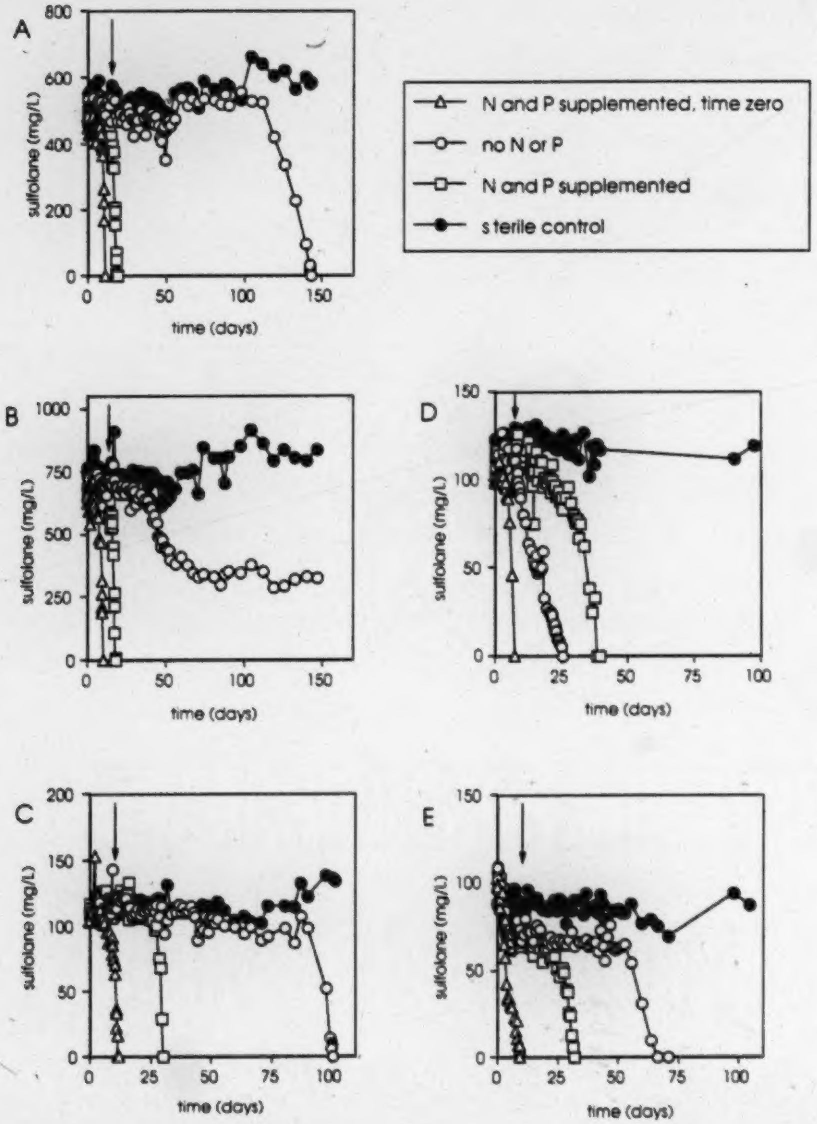


Figure 3.7. Loss of sulfolane in shake-flask cultures inoculated with samples taken from site 2: aquifer sediments and the corresponding groundwaters or wetlands materials and the corresponding surface waters and incubated at 10°C. A. East contaminated aquifer. B. West contaminated aquifer. C. Uncontaminated aquifer. D. West wetland. E. East wetland. Arrows indicate the time at which cultures that had been modified with 3.1 mg/L N and 79 mg/L P at time zero were modified with 31 mg/L N and 790 mg/L P. A diagram of the sampling sites is given in Figure 2.1.

organic material in the form of plant matter; therefore the likely explanation is that sufficient N and P for sulfolane biodegradation were available from the organic materials present in this sample. If this was the case, N and P supplementation would have little effect on sulfolane biodegradation in these cultures, and the shorter lag time for the unsupplemented culture was probably due to variability between samples. The faster rate of sulfolane removal with supplementation of a high level of N and P at time zero (Figure 3.7D) supports this hypothesis, however none of these hypotheses were tested.

With the exception of the culture inoculated with materials from the west wetland region, the lag times were shorter in all the N- and P-supplemented cultures than in the unsupplemented cultures, particularly when a high level of N and P was added at time zero. Only the east plume and west plume cultures showed evidence of some sulfolane biodegradation when the lower concentration of nutrients (3.1 mg/L N and 79 mg/L P) were present (Figure 3.7A, B). However, the additional supplementation with N and P greatly stimulated sulfolane biodegradation in all the cultures (Figure 3.7). After the N and P concentration had been augmented, the lag times varied from 10 d in the shake flasks containing uncontaminated sediment or east wetland material to 14 d in the culture inoculated with mud from the west wetland (Figure 3.7C-E). The shortest lag times were observed in cultures supplied with 31 mg/L N and 790 mg/L P at time zero (Figure 3.7, Table 3.7), demonstrating that high levels of nutrient supplementation had the most stimulatory effect on sulfolane biodegradation. The longest lag time before sulfolane degradation occurred in the unsupplemented cultures inoculated with uncontaminated aquifer sediment (Figure 3.7, Table 3.7). In the N- and P-supplemented cultures, the longest lag times were observed for the cultures inoculated with uncontaminated aquifer sediment or wetland materials. The overall conclusion for aquifer sediment samples was therefore that when cultures were supplemented with N and P, previous exposure of the inocula to sulfolane appeared shorten the lag time before biodegradation commenced. These data are supported by the initial population screening experiments (Section 3.1.2), where uncontaminated soil or sediment cultures showed lag times 7- to 17-fold longer than contaminated aquifer sediment cultures. This difference in the lag times was likely due to the presence of a sulfolane-degrading microbial population in the contaminated aquifer sediments; such a population would have to develop in the uncontaminated sediments after the experiment began, when the microbial population was first exposed to sulfolane.

Table 3.7. Sulfolane biodegradation in shake-flask cultures inoculated with aquifer sediment or wetland materials from five sampling points at site 2. Cultures were incubated at 10°C.

sample	N and P added	total sulfolane removed (mg/L)	% of total sulfolane removed	lag time (d)	biodegradation rate (mg/L/h)
east plume	Y[a]	450	100	8	6.0
	Y[b]	520	100	13	3.7
	N	540	100	98	0.6
west plume	Y[a]	630	100	8	10.3
	Y[b]	720	100	12	5.2
	N[c]	370	52	34	0.4
uncontaminated	Y[a]	100	100	9	1.4
	Y[b]	120	100	24	1.7
	N	110	100	88	0.4
west wetland	Y[a]	100	100	4	1.1
	Y[b]	110	100	28	0.3
	N	120	100	9	0.2
east wetland	Y[a]	99	100	2	1.1
	Y[b]	110	100	24	0.3
	N	110	100	53	0.2

[a] 31 mg/L N and 790 mg/L P added at time zero.

[b] 3.1 mg/L N and 79 mg/L P added at time zero; supplemented to 31 mg/L N and 790 mg/L P at 14 d.

[c] Removal of sulfolane from the west plume culture that was not supplemented with N or P was not complete; in this culture the kinetics of degradation appeared to be first-order rather than zero-order, however the zero-order kinetic rate of biodegradation is reported here for the purpose of comparison.

The initial sample taken to determine the sulfolane degradation potential at site 2 (Section 3.1.2) was taken from a site downgradient from the east plume sample used for the nutrient requirement study. The original sample was taken in the same sulfolane concentration range as the east plume sample, and well short of the wetlands region. The lag times for sulfolane degradation in the shake-flask cultures incubated for the initial population screening study were 6 and 9 d (Table 3.1), which was consistent with the data for both the east plume and west plume cultures incubated for this set of experiments, both of which had lag times of 8 d (Table 3.7). This information suggests that the use of groundwater as the aqueous portion of the cultures, rather than mineral medium, did not have a strong effect on lag times before biodegradation commenced.

Kinetic analyses of the data for N- and P-supplemented cultures revealed that once biodegradation started, almost all sulfolane loss followed zero-order kinetics (Table B2, Appendix B). The degradation rates for N- and P-supplemented cultures varied from 3.7 to 10.3 mg/L/h for the contaminated east plume and west plume cultures, 1.4 to 1.7 mg/L/h in the uncontaminated culture and 0.3 to 1.1 mg/L/h in both the east wetland and west wetland cultures (Table 3.7). These data suggest that previous exposure to high sulfolane concentrations may have enhanced the ability of the microbial community to degrade this compound, at least in the aquifer sediment cultures. For the wetland cultures, it is also possible that microbial activity was directed toward other organic C sources than sulfolane, which would be liable to decrease the rate of sulfolane biodegradation.

The cultures incubated in the initial study (Section 3.2.1) showed sulfolane degradation rates of 2.7 and 2.0 mg/L/h in the cultures incubated at 8°C (Table 3.1). The rates of sulfolane degradation were therefore noticeably lower in the initial study. This difference in rate may be due to the higher sulfolane concentrations in aquifer sediments incubated with groundwater (2- to 3- fold higher, and 493 and 675 mg/L for the east and west plumes, respectively, compared with 200 mg/L in the initial experiment), however it was not possible to confirm this hypothesis. As the site had been contaminated with sulfolane for > 30 y, it is unlikely that a large change in the microbial population on site occurred over the time between the two sampling dates (approximately 1.5 y). It is also unlikely that the slight difference in sampling location contributed significantly to the observed differences in biodegradation rate.

Neilsen and Christensen (1994) demonstrated that the biodegradation of some aromatic compounds did not differ substantially in samples removed from different parts of a landfill site that were defined by the same hydrogeology, however samples from a region of the landfill that demonstrated differences in groundwater flow showed different microbial activity against the compounds. As the samples in this study were taken from a region with the same hydrogeology and groundwater source (Figure 2.1), it was predicted that the microbial activity in both samples would be similar. Holm et al. (1992) observed microbial activity against xenobiotic compounds in groundwater in the absence of sediment; it is possible that the increased biodegradation rate observed between the initial population screening experiments, which did not contain groundwater, and the current shake-flask experiments was due to the microbial population contributed by the groundwater samples. Xenobiotic biodegradation by groundwater, however, occurred much more slowly than by sediment microorganisms (Holm et al., 1992), therefore such a contribution might not completely explain the difference in biodegradation rates observed here.

It was interesting that shake-flask cultures inoculated with uncontaminated aquifer sediment incubated with the corresponding groundwater sample demonstrated much lower rates of sulfolane degradation (0.4 to 1.4 mg/L/h; Table 3.7) than those inoculated with the east plume and west plume contaminated aquifer sediments (0.6 to 6.0 mg/L/h and 0.4 to 10.3 mg/L/h, respectively; Table 3.7). In the previous experiment where the sulfolane degradation rates of contaminated and uncontaminated sediments were compared, the rate of sulfolane degradation was similar in both types of sediment, although the lag times were much longer with the uncontaminated sediment (Table 3.1). It is possible that the groundwater which was used as the aqueous portion of the uncontaminated sediment cultures lacked a trace nutrient that was added to the previous cultures, which consisted of aquifer sediment in mineral medium. It is also likely that the incubation temperature contributed to the higher degradation rates seen in the previous cultures; they were incubated at 28°C, rather than at 8°C, and a wider variety of microorganisms may have been active at the higher temperature.

Degradation rates for the unsupplemented cultures were generally lower than those for the N- and P-supplemented cultures, ranging from 0.2 mg/L/h for the east wetland and west wetland cultures to 0.6 mg/L/h for the east plume culture (Table 3.7). As with the N- and P-supplemented cultures, degradation generally followed zero-order kinetics. The only exception was the west plume culture, where the data fitted first-order kinetics more closely than zero-order kinetics (Table B2, Appendix B). However, the loss of sulfolane in this culture was not complete (Figure 3.7), thus this kinetic description may not be accurate with respect to complete removal of sulfolane from a sample of this aquifer material.

Figure 3.8 summarizes the relationship between removal of sulfolane from selected shake-flask cultures and the pH of the cultures. In most cases the biodegradation of sulfolane was accompanied by a drop in pH; this pH decrease has previously been observed by other researchers (Chou and Swatloski 1983). Aerobic sulfolane biodegradation releases acid, as described by the equation in Table 3.5.

Treatment of the three contaminated and one uncontaminated aquifer sediments with 0.1 M HCl resulted in effervescence, suggesting the presence of carbonates in these samples. Carbonate in these sediments could provide buffering for the acid produced during sulfolane degradation. Presuming that the buffering capacities of the three aquifer sediments used in this study were similar, one would expect that the higher the initial sulfolane concentration in the shake-flask culture, the greater the pH drop would be after the sulfolane was completely degraded in these batch cultures. The initial sulfolane concentrations in the west plume and east plume shake-flasks cultures were 680 mg/L and 490 mg/L, respectively, whereas 120 mg/L sulfolane was added to the shake-flask cultures that contained uncontaminated sediment. Despite these differences in sulfolane concentrations, there was no clear trend demonstrated by the pH decreases (data not shown). The pH drop in the cultures varied between 0.1 and 2.0 pH units which may have been too small for accurate observations. However, it is likely that the most important factor governing pH changes in the aquifer sediment cultures was the buffering potential offered by the sediment itself. The carbonates in these clay-containing aquifer sediments likely provided buffering in cultures containing these sediments.

Factors other that sulfolane biodegradation may also have affected the pH of the shake-flask cultures; a marked increase in pH after the initial pH drop was observed in some cultures, particularly in the N- and P-supplemented cultures containing west plume sediment (Figure 3.8A) or uncontaminated sediment (data not shown). The pH rose approximately 1 pH unit in the former culture and about 1.8 pH units in the latter culture. The cause of these increases is unknown, however it is possible that biodegradation of DIPA, which was also present in both cultures, may have resulted in the release of ammonia (Gieg et al., 1999). It is unlikely that compounds other than DIPA were involved in the pH increase in the uncontaminated sediment as very little organic C was detected in this sample (< 1 mg/L C in the corresponding groundwater).

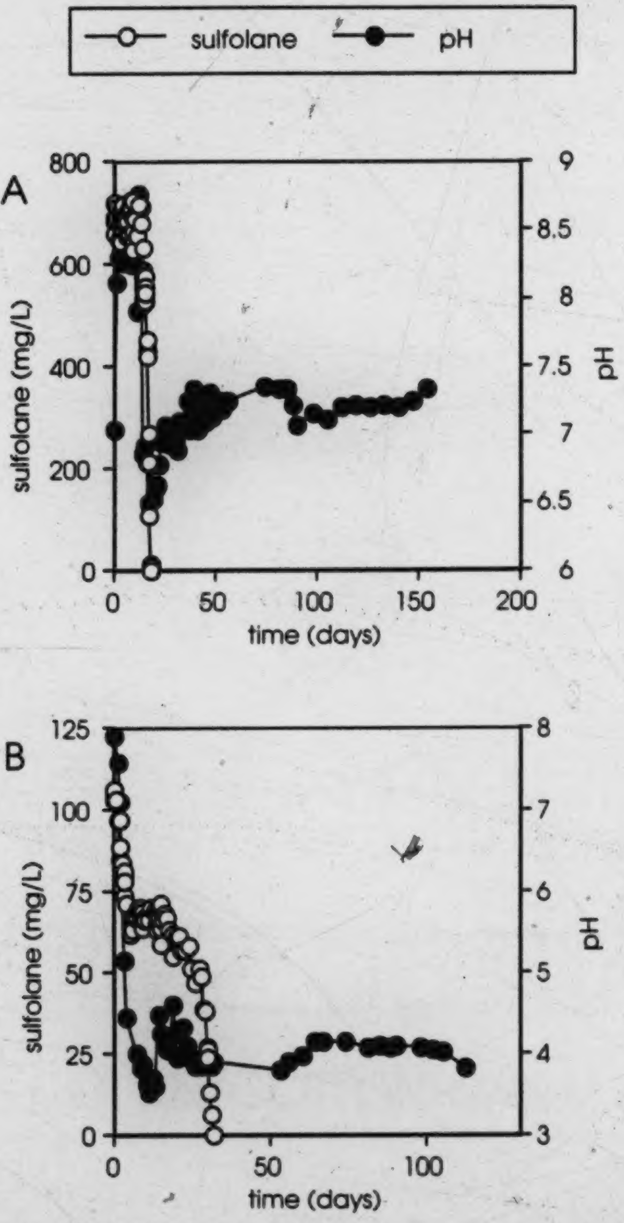


Figure 3.8. Loss of sulfolane from shake-flask cultures inoculated with site 2 aquifer sediment or wetland material incubated at 10°C, and the corresponding change in pH. A. West plume aquifer sediment modified with 3.1 mg/L N and 79 mg/L P at time zero, then 31 mg/L N and 790 mg/L P at 14 d. B. East wetland material modified with 3.1 mg/L N and 79 mg/L P at time zero, then 31 mg/L N and 790 mg/L P at 14 d.

At the onset of sulfolane biodegradation in the shake-flask cultures that contained east wetland material, was a sharp drop in pH (Figure 3.8B). In these cultures, sulfolane biodegradation stalled when the pH fell below 4. The microorganisms in these shake-flask cultures appeared to adapt to this low pH; after an acclimation period sulfolane biodegradation resumed, and all of this substrate was eventually removed from these cultures. While the aquifer sediments were high in clay materials, which were likely to contain mineral components, such as carbonate, that greatly increased the buffering capacity of the shake-flask cultures, wetland materials were high in organic matter and contained little clay, therefore were less likely to have a good buffering capacity. This prediction was proven by the decrease in pH of approximately 4 pH units observed in the east wetland cultures. Although the drop in pH may have temporarily stalled microbial activity in these cultures, the community appeared to be capable of adapting to the change. A similar pH drop would not be expected to occur in the field; the wetland system would be much more complex, and acidic products would be likely to migrate with ground and surface water, becoming diluted as they were produced.

Four conclusions can be drawn about aerobic sulfolane biodegradation at site 2, based on these shake-flask experiments.

1. Cultures derived from uncontaminated sediment were able to biodegrade sulfolane, although a longer lag time and a lower rate of degradation were observed than for acclimated sediments. This observation is supported by the longer lag times observed for uncontaminated soils in the initial population screening experiment (Section 3.2.1); the low degradation rate for unacclimated soils was not previously observed, however this is likely accounted for by the differences in incubation conditions between the two experiments.
2. The shortest lag times before biodegradation commenced in sediment cultures were generally observed in the cultures comprised of sediments and groundwaters previously exposed to high concentrations (>400 mg/L) of sulfolane.
3. Inocula from the wetland regions, which have been exposed to lower levels of sulfolane than the aquifer sediments, also demonstrated relatively short lag times before biodegradation commenced when compared with uncontaminated soils. This may be due to diversity of the microbial community present in the wetland regions, which contained natural organic material, including large pieces of vegetative matter. This high organic content would provide conditions for a large and diverse microbial community to have developed in the wetlands.

4. Supplementation of the cultures with N and P decreased the lag time before sulfolane degradation occurred, and increased the rate of biodegradation. In one case, with the west plume contaminated aquifer sediment, complete removal of sulfolane did not occur in the absence of N and P supplementation. Because N and P were added to the shake-flask cultures in this experiment simultaneously, it was not possible to tell which of the two nutrients was most limiting for sulfolane degradation.

It should be noted that these experiments involved a high level of aeration and mixing, therefore reported rates of biodegradation were substantially higher than those that would be observed in groundwaters or aquifers, even in areas where there were significant concentrations of dissolved O_2 in the groundwater.

3.3.2 N- and P-supplemented shake-flask cultures

The first series of shake-flask cultures used to test the effects N and P supplementation on sulfolane biodegradation demonstrated that the presence of these nutrients decreased the lag time and increased the degradation rate for sulfolane removal by the microbial population of aquifer sediments (Section 3.3.1). The relative importance of N and P on sulfolane biodegradation in the aquifer sediments was not elucidated; therefore this experiment was designed to determine which of the two nutrients was more rate limiting in the two contaminated groundwater and aquifer sediment samples. The wetland materials did not suffer from nutrient limitation with respect to sulfolane biodegradation, and the uncontaminated aquifer sediment had no sulfolane present, therefore these samples were not used in this experiment. The amount of each nutrient required to account for all the sulfolane and DIPA in the west plume groundwater was calculated based on the C:N:P ratio of 100:5:1 (Alef and Nannipieri 1995). The concentrations of N and P required to achieve this C:N:P ratio were calculated assuming that no source of N or P was present in the sediment and groundwater samples, and the resulting concentrations of N and P were defined as N_r and P_r, respectively. Zero, 1-fold or 2-fold this amount of each inorganic nutrient was added to cultures comprised of sediment and groundwater from the west contaminated plume to test their effects on biodegradation. The same amount of N and P was added to cultures comprised of sediment and groundwater from the east contaminated plume. This experiment is described in Section 2.7.2.2.

In groundwater taken from the west contaminated plume, sulfolane and DIPA contributed 310 mg/L C, therefore N_r was 16 mg/L and P_r was 3.1 mg/L. For the east

Table 3.8. The amount of N and P added to each culture in order to determine the overall importance of each of these nutrients to sulfolane biodegradation in shake-flask cultures comprised of aquifer sediments from the site 2 east and west contaminated plumes. The concentrations of N and P required to achieve a C:N:P ratio of 100:5:1 were defined as N_r and P_r, respectively.

west plume[a]			east plume[b]		
$0N_r$	$1N_r$	$2N_r$	$0N_r$	$0.8N_r$	$1.6N_r$
$0P_r$	$0P_r$	$0P_r$	$0P_r$	$0P_r$	$0P_r$
$1P_r$	$1P_r$	$1P_r$	$0.8P_r$	$0.8P_r$	$0.8P_r$
$2P_r$	$2P_r$	$2P_r$	$1.6P_r$	$1.6P_r$	$1.6P_r$
sterile[c]			sterile[c]		$2.4P_r$
					$3.2P_r$
					$4.0P_r$

[a] A total of nine viable shake-flask cultures were incubated.

[b] A total of twelve viable shake-flask cultures were incubated.

[c] sterile control: no N or P added.

contaminated plume groundwater, sulfolane and DIPA contributed 386 mg/L C; N_r was 19.3 mg/L and P_r was 3.9 mg/L. The amount of N and P added to the east plume cultures was therefore 0, 0.8, and 1.6 N_r and/or P_r. The highest P concentration tested was 32 mg/L P, corresponding to $255P_r$ in the west plume cultures and $205P_r$ in the east plume cultures, which was the same concentration of P used in the initial shake-flask cultures used to determine the sulfolane biodegradation potential at this site (Sections 3.2.1 and 3.3.1). The N and P concentrations tested for the east and west plume aquifer sediments are summarized in Table 3.8.

Due to the constraints of available materials, shaker space and analytical time, single cultures were incubated for each condition that was tested. Previous shake-flask experiments using materials from this site demonstrated good reproducibility between duplicate cultures (Table 3.1, Section 3.2.1). This series of experiments was carried out using various combinations of N and P concentrations that would have exposed anomalous results, therefore duplicate cultures were not deemed necessary. Furthermore, Neilsen and Christensen (1994) performed a series of experiments with materials from eight sampling sites, using duplicate microcosms for each site, and for all tests, the authors found that the results from the duplicate microcosms were consistent.

3.3.2.1 West contaminated plume

In the west plume cultures, sulfolane biodegradation was more dependent on P than on N concentration (Figure 3.9, Table 3.9). Both the lag times before commencement of sulfolane degradation and the rate of decrease of the compound exemplified this dependence. Cultures that received no added P had lag times of 20 to 22 d, regardless of the amount of N added (Figure 3.9, Table 3.9). The addition of $1P_r$ or $2P_r$ to the cultures decreased the lag times before sulfolane degradation to between 9 and 13 d. Overall, little or no difference was observed between the lag times for samples containing various amounts of N and the same amount of P when compared with the lag times for samples containing various amounts of P and the same amount of N.

Similarly, supplementation with P had a greater influence on the sulfolane zero-order biodegradation rates than supplementation with N in the west plume shake-flask cultures (Figure 3.9, Table 3.9). With no added P, biodegradation rates were between 1.0 and 1.4 mg/L/h, regardless of the amount of N added. Supplementing the cultures with $1P_r$ caused an approximately 3-fold increase in the rate of sulfolane biodegradation, while $2P_r$ increased the rate approximately 5-fold. At this P concentration, supplementing with $1N_r$ and $2N_r$ increased the sulfolane biodegradation rate to 10 and 12 mg/L/h, respectively (Figure 3.9, Table 3.9), therefore when P was abundant, N may have become limiting to the sulfolane biodegradation rate.

3.3.2.2 East contaminated plume

The lag times and biodegradation rates for shake-flask cultures that contained east plume materials are summarized in Table 3.9. As observed with the west plume cultures, sulfolane biodegradation was more dependent on supplementation with P than with N in these cultures (Figure 3.10, Table 3.9). As a general rule, the biodegradation rates increased with increasing P concentration up to $4P_r$. From $0P_r$ to $4P_r$, there was a strong linear correlation between the rates of sulfolane biodegradation and the P concentrations in the shake-flask cultures (data not shown). It appeared that P was not limiting to the rate of sulfolane biodegradation at concentrations of $3.2P_r$ and greater (Table 3.9). Therefore P was the limiting factor for sulfolane biodegradation in these cultures. This limitation appeared to follow the substrate saturation curve described by Michaelis-Menten kinetics (Matthews and van Holde, 1990), although more data points would be required to substantiate this hypothesis.

For comparable amounts of P supplementation, the rates of sulfolane biodegradation in the shake flasks containing materials from the west plume were greater than those in shake flasks containing materials from the east plume. For example, when supplemented with 3.1 mg/L P ($1P_r$ in the west plume cultures, $0.8P_r$ in the east plume cultures), the rates of sulfolane degradation in the west plume cultures were 3.8 to 4.4 mg/L/h whereas the rates in the east plume cultures were 1.5 to 2.1 mg/L/h. Similarly, in cultures that were not supplemented with N but received 6.2 mg/L P ($2P_r$ in the west plume cultures, $1.6P_r$ in the east plume shake-flask cultures), the rate of sulfolane degradation in the west plume culture was 6.3 mg/L/h whereas the rate in the east plume culture was 3.0 mg/L/h. These differences in rates may be attributed to the differences in total organic C concentrations in the two groundwater samples. The east plume groundwater contained 650 mg/L organic C, of which 202 mg/L came from sulfolane while the west plume groundwater contained 550 mg/L organic C, of which 297 mg/L came from sulfolane (Table 2.2). The higher organic C concentration in the east plume groundwater may have exerted a higher demand for P for the biodegradation of compounds other than sulfolane, thereby causing a lower sulfolane biodegradation rate in the east plume shake-flask cultures; for instance DIPA represented 188 mg/L of the total organic C in the east plume cultures and only 38 mg/L of the C in the west plume cultures. Degradation of DIPA would release N (Gieg et al., 1999), which would be

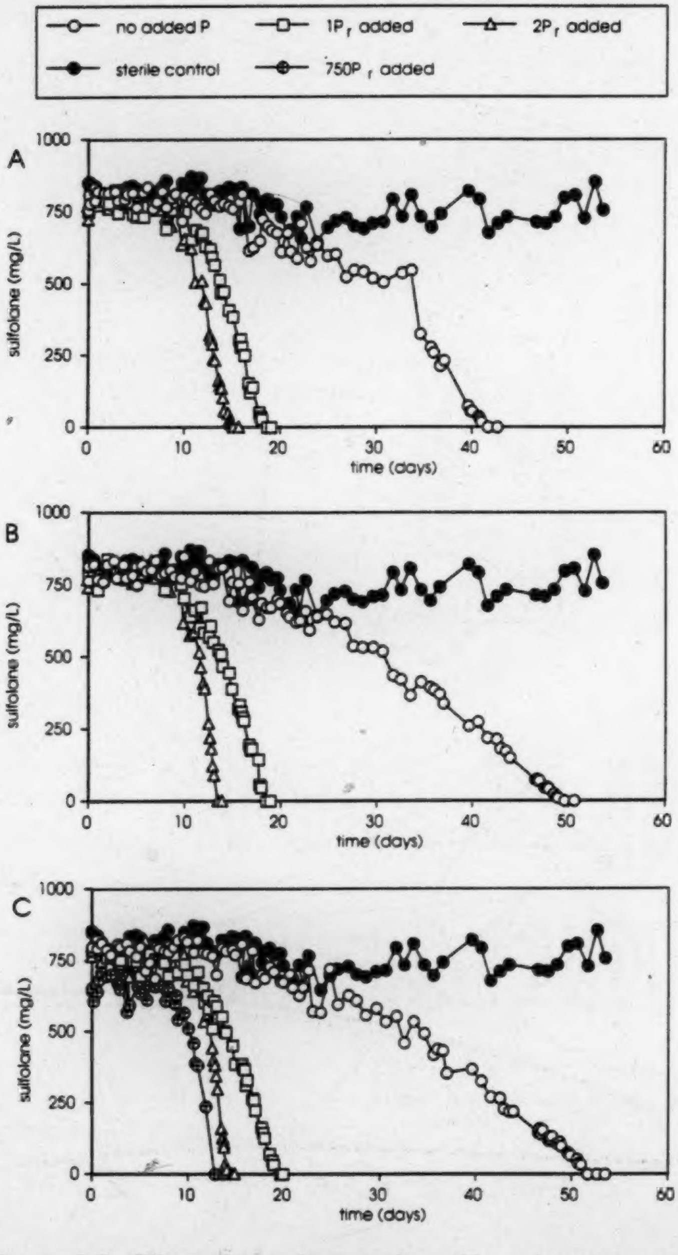


Figure 3.9. Effect of supplementation with various amounts of N or P on sulfolane biodegradation in cultures consisting of site 2 west plume aquifer sediment and groundwater. The cultures were incubated at 10°C. The appellations N_r and P_r represent the concentrations of N and P required to achieve a C:N:P ratio of 100:5:1. A. Various P_r with $0N_r$. B. Various P_r with $1N_r$. C. Various P_r with $2N_r$.

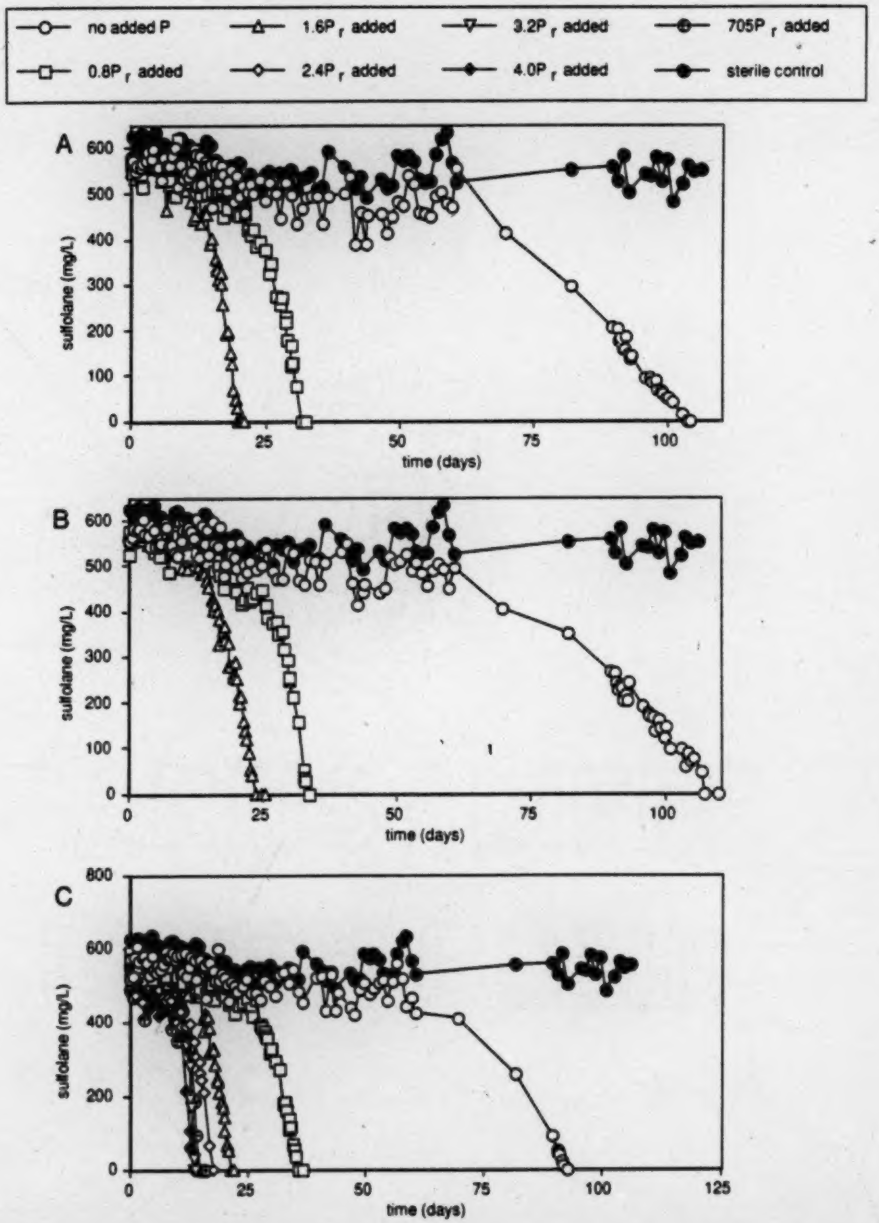


Figure 3.10. Effect of supplementation with various amounts of N or P on sulfolane biodegradation in cultures consisting of site 2 east plume contaminated aquifer sediment and groundwater. The cultures were incubated at 10°C. The appellations N_r and P_r represent the concentrations of N and P required to achieve a C:N:P ratio of 100:5:1 A. Various P_r with $0N_r$. B. Various P_r with $0.8N_r$. C. Various P_r with $1.6N_r$.

Table 3.9. Biodegradation of sulfolane in shake-flask cultures consisting of aquifer sediment and groundwater from the east or west contaminated plumes supplemented with various concentrations of N and P. Cultures were incubated at 10°C. No loss of sulfolane was observed in any of the sterile control cultures. The concentrations N_r and P_r represent the amount of N and P required to achieve a C:N:P ratio of 100:5:1.

west plume			east plume		
N and P added	lag time (d)	rate (mg/L/h)	N and P added	lag time (d)	rate (mg/L/h)
$0N_r$, $0P_r$	22	1.4	$0N_r$, $0P_r$	58	0.5
$1N_r$, $0P_r$	20	1.0	$0.8N_r$, $0P_r$	58	0.4
$2N_r$, $0P_r$	21	1.0	$1.6N_r$, $0P_r$	57	0.6
$0N_r$, $1P_r$	23	4.4	$0N_r$, $0.8P_r$	21	1.5
$1N_r$, $1P_r$	12	3.9	$0.8N_r$, $0.8P_r$	19	2.1
$2N_r$, $1P_r$	13	3.8	$1.6N_r$, $0.8P_r$	27	2.0
$0N_r$, $2P_r$	20	6.3	$0N_r$, $1.6P_r$	14	3.0
$1N_r$, $2P_r$	9	10	$0.8N_r$, $1.6P_r$	12	1.8
$2N_r$, $2P_r$	12	12	$1.6N_r$, $1.6P_r$	13	2.9
$2N_r$, $255P_r$	10	6.2	$1.6N_r$,$2.4P_r$	13	3.0
			$1.6N_r$,$3.2P_r$	8	4.6
			$1.6N_r$,$4.0P_r$	9	4.8
			$1.6N_r$, $205P_r$	15	4.5

available for sulfolane-degrading microbes, but would likely consume some of the available P in the cultures simultaneously. Conversely, the higher amount of C initially found in the east plume groundwater could have been the result of lower microbial activity.

3.3.2.3 Summary of biological activity in the shake-flask cultures with various concentrations of N and P

The lag times and biodegradation rates documented in the shake-flask cultures supplemented with various amounts of N or P clearly demonstrated that the biodegradation of sulfolane by microbial populations from aquifer sediments located in the east and west contaminated plumes at site 2 was limited by the supply of P. In the west plume cultures, if sufficient P ($2P_r$) was added then an increase in N appeared to further increase the rate of sulfolane degradation, however the presence of N did not appear to affect the lag time before sulfolane biodegradation. In these cultures, the rate of DIPA degradation increased and the lag time before degradation commenced decreased with increasing N concentrations when cultures were supplemented with either $1P_r$ or $2P_r$; no similar improvements in DIPA removal were observed in the absence of added P (Greene et al., submitted).

Braddock et al. (1997) demonstrated that N was the major limiting factor for hydrocarbon biodegradation by the microbial population of an Arctic soil, however P, to a lesser extent, also played a role in nutrient limitation; both nutrients were required to maximize the degradation rate, which was similar to the findings reported here. Because DIPA biodegradation releases N (Gieg et al., 1999) that could be utilized by sulfolane-degrading microbes, it is reasonable to suggest that any small effects on sulfolane biodegradation by the addition of N might be masked by N release from DIPA. The minimum requirement for addition of N and P to this site for bioremediation could not be calculated; field tests involving air sparging combined with nutrient supplementation, or landfarming, would be required to determine this information. The information provided by this experiment, however, have provided a starting point from which the amounts of N and P added to pilot scale field experiments could be estimated.

Grainger (1997) used aerobic laboratory microcosms to examine the relative roles of N and P supplementation in biodegradation of BTEX and some trimethylbenzenes. Microcosms that were amended with N but not P showed negligible biodegradation over the 62-d incubation period, whereas the lag times and substrate utilization rates in the microcosms amended solely with P were similar to those observed in microcosms supplemented with both N and P (Grainger, 1997). These findings were similar to the results obtained for sulfolane degradation in the site 2 aquifer sediment from the east and west contaminated plumes. Most authors reviewed here (Section 1.9) demonstrated a requirement for N rather than for P, at least as the most important nutrient involved in limitation of biodegradation at a site. The results of Grainger (1997) and the P requirement observed in site 2 contaminated sediments demonstrate that nutrient requirements can be dependent on the contaminated site, and may not follow the general trends published in the literature. It is possible that in sites where DIPA and other N-containing compounds comprise a significant portion of the contaminant compounds, less N addition may be required for biodegradation of organic C compounds.

In general, the minimum concentrations of N and P required for biodegradation at a given site will vary, depending on the nutrient requirements at the location. As well as varying between sites, nutrient requirements will vary on the site. The major effect will probably be due to differences in organic C concentrations; thus the C:N:P ratio will be more important than the absolute amount of N and P available at a given location.

3.3.3 2.5-L microcosms

Design of the 2.5-L microcosms was based on the experimental setup described by Holm et al. (1992) for *in situ* and laboratory microcosms (described in Section 2.7.2.3). A similar design was also employed by Nielsen and Christensen (1994). Both authors observed a good correlation between laboratory and *in situ* microcosms using this experimental design; Holm et al. (1992) observed activity against a broader spectrum of xenobiotic compounds *in situ* than in the laboratory. For the intent of this experiment, the 2.5-L microcosms were used to compare results observed in laboratory shake-flask tests with an experimental setup that more accurately reflected conditions that would be observed in an aerobic aquifer in the field.

A total of nine microcosms were incubated: east plume, west plume, east wetland and west wetland viable microcosms and the corresponding sterile control microcosms, and an uncontaminated aquifer sediment viable microcosm. Due to the limited quantity of the uncontaminated aquifer sediment and groundwater samples, no sterile control microcosm was made. Prior to combining the appropriate sediments and water samples in the 2.5-L microcosms, the water samples were vigorously aerated. The microcosms were shaken at 40 rpm; at this slow shaking speed, the sediments settled during the first few days of incubation, leaving a relatively clear layer of water about 10 cm deep in the wetland microcosms and 15 cm deep in the aquifer sediment microcosms. The shaking speed was sufficient to maintain aerobic conditions in the water layer of the microcosms.

Removal of sulfolane to below detectable levels occurred in all the viable microcosms except the one inoculated with uncontaminated sediment and groundwater (Figure 3.11). The microcosms consisting of wetland materials degraded sulfolane relatively quickly (Figure 3.11D, E). Little loss of sulfolane was observed in the east and west plume microcosms until after the addition of 1 P_r at 221 d; after this supplementation sulfolane degradation was observed in both microcosms (Figure 3.11A, B). Shake-flask experiments (Section 3.3.2) demonstrated that P was the limiting factor for sulfolane degradation in these aquifers, therefore P was added to the microcosms to determine whether sulfolane degradation could be stimulated. During an incubation period of 377 d, no significant removal of sulfolane from the uncontaminated aquifer sediment microcosm was observed (Figure 3.11C). At 221 d, 1P_r was added to the uncontaminated microcosm, and at 294 d, 1P_r and 1N_r were added to the microcosm. The lack of activity in this microcosm suggests that it may take more than 1 y for a sulfolane-degrading population to develop in a pristine aquifer. It is unlikely that nutrient requirements, other than the presence of O_2 or incubation requirements other than mixing, would have affected sulfolane biodegradation in this microcosm; shake-flask cultures containing the same sediment and groundwater inoculum were able to degrade sulfolane (Figure 3.8, Table 3.7). The shake-flask cultures also demonstrated the existence of microorganisms capable of developing sulfolane-degrading abilities in this aquifer sediment.

The dissolved O_2 concentrations in microcosms that contained aquifer sediments were stable between about 7.5 mg/L and 10 mg/L during the experiment (Figure 3.11A-C). Dissolved O_2 in the west wetland microcosm remained at approximately 8 mg/L throughout the experiment, although some variability was observed (Figure 3.11). In the east wetland microcosm, dissolved O_2 decreased sharply early in the incubation period.

In the west wetland 2.5-L microcosm, a significant drop in the O_2 level occurred at approximately 32 d, and another at approximately 53 d. In both cases the dissolved O_2 level returned to near 8 mg/L without any intervention such as reaeration of the microcosm. It is unlikely that these decreases were artifacts of the experiment; each decrease in dissolved O_2 persisted over more than one sampling time. A likely explanation is that biodegradation of organic materials at various times may have

Table 3.10. Biodegradation of sulfolane in 2.5-L microcosms consisting of aquifer sediment and groundwater from the east or west contaminated plumes or uncontaminated region, or wetland material and surface water from the east or west wetland regions. Microcosms were incubated at 10°C. At 221 d, $1P_r$ was added to the east plume, west plume and uncontaminated aquifer sediment microcosms. At 294 d, $1N_r$ and a second dose of $1P_r$ were added to the uncontaminated aquifer sediment microcosm. The concentrations N_r and P_r represent the amount of N and P required to achieve a C:N:P ratio of 100:5:1.

microcosm identity	lag time (d)	degradation rate (mg/L/h)
east plume	225	0.5
west plume	249	0.3
uncontaminated	>377	0.0[a]
west wetland	39	0.3
east wetland	25	0.4

[a] No significant loss of sulfolane was observed over the 377-d incubation period.

resulted in a temporary decrease in dissolved O_2 in the microcosm, during which time the rate of O_2 diffusion into the aqueous groundwater layer was insufficient to keep up with the rate of O_2 consumption, but that each increase in O_2 utilization was of short duration and O_2 was soon replenished to the steady state concentration.

The amount of O_2 required for sulfolane biodegradation can be calculated, using the equation for the complete oxidation of sulfolane under aerobic conditions (Table 3.5). Sulfolane biodegradation in the west wetland microcosm occurred at a rate of 0.3 mg/L/h (Table 3.8), or 2.5 µmol/L/h. Over the time biodegradation of sulfolane occurred 100 mg/L (0.83 mmol/L) was removed from the microcosm. According to the equation for sulfolane oxidation under aerobic conditions, this amount of sulfolane oxidation would require 5.4 mmol/L O_2. The dissolved O_2 concentration in the microcosm was approximately 7.5 mg/L (0.23 mmol/L). As sulfolane biodegradation was observed in this microcosm, and there was not enough dissolved O_2 to satisfy this demand at any single time during sulfolane degradation, reoxygenation must have occurred during the incubation. The sulfolane biodegradation rate of 2.5 µmol/L/h suggests that the rate of O_2 consumption was at least 16.2 µmol/L/h, therefore the rate of reoxygenation would have had to be $\geq$ 16.2 µmol/L/h. It is likely that shaking the microcosms at 40 rpm was sufficient to allow reoxygenation of the aqueous portion of the microcosms at this rate.

A sharp decrease in dissolved O_2 occurred in the east wetland microcosm at 7 d (Figure 3.11E). This coincided with a decrease in sulfolane concentration of about 20 mg/L (0.17 mmol/L) that occurred at a rate of 0.69 mg/L/h (5.8 µmol/L/h). Considering the data from the west wetland microcosm, the reoxygenation rate may not have provided sufficient O_2 for sulfolane biodegradation in the east wetland microcosm at this time, leading to the marked depletion of dissolved O_2. At 15 d, the microcosm was reoxygenated through 6 h of forced aeration; however this prolonged aeration did not increase the dissolved O_2 concentration to more than 2 mg/L. Re-aeration resulted in the resuspension of the solids in the east wetland microcosm. Interestingly, as the solids settled in this microcosm after aeration, the dissolved O_2 content rose to approximately 8 mg/L and remained stable at this level for the remainder of the experiment. After reoxygenation of the microcosm, sulfolane biodegradation occurred within 10 d and the majority of the compound was removed without a decrease in the dissolved O_2 concentration. Although sulfolane biodegradation may have contributed to the decrease in dissolved O_2 in the east wetland microcosm, it likely did not account for the total O_2 depletion observed; other organic C sources, including DIPA and plant material, were

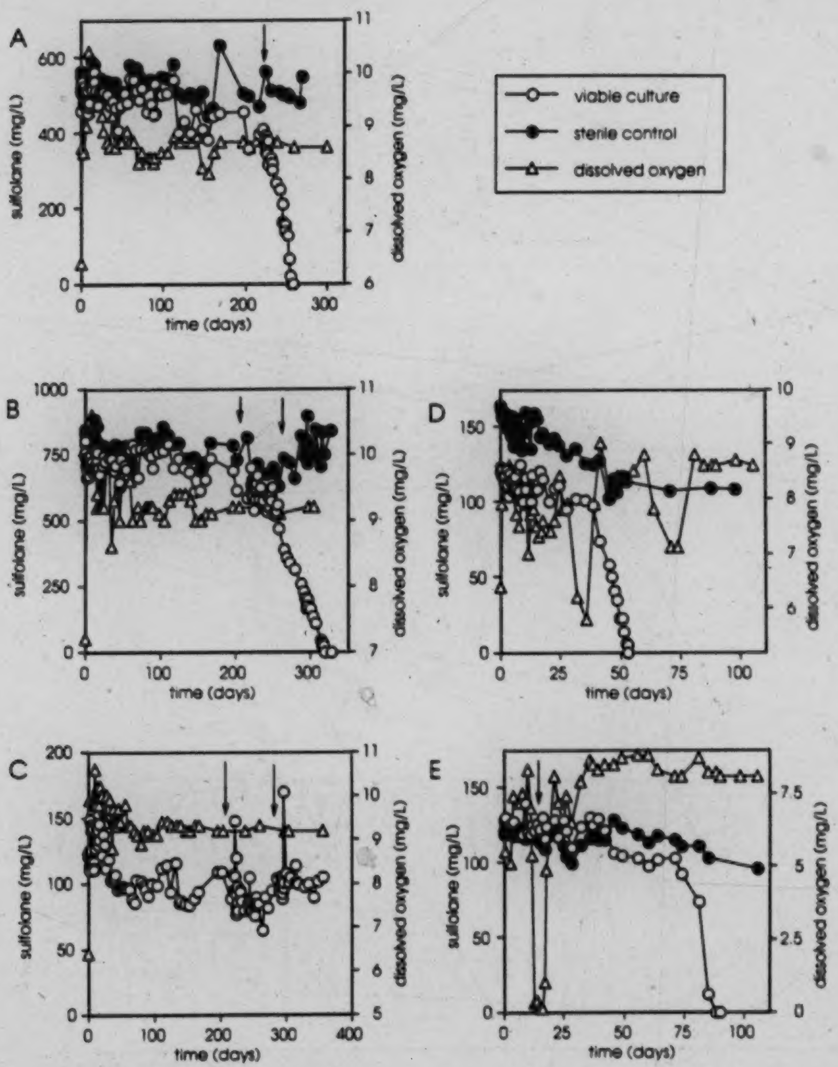


Figure 3.11. Biodegradation of sulfolane and the dissolved O_2 concentration in 2.5-L microcosms incubated at 10°C. No dissolved O_2 measurements were made in the sterile microcosms. A. East contaminated plume. The arrow indicates the addition of $1P_r$ at 221 d. B. West contaminated plume. The arrow indicates the addition of $1P_r$ at 221 d. C. Uncontaminated sediment. The first arrow indicates the addition of $1P_r$ at 221 d. The second arrow indicates the addition of $1N_r$ and $1P_r$ at 294 d. D. West wetland material. E. East wetland material. The arrow indicates reoxygenation of the microcosm by forced aeration at 15 d.

also present. However it is likely that mixing played a role in the decrease in sulfolane concentration subsequent to reaeration of the microcosm.

Lag times before sulfolane biodegradation commenced ranged from 25 d and 39 d in the east and west wetland microcosms, respectively, through 225 d and 249 d for the east and west plume microcosms, respectively to > 377 d for the uncontaminated microcosm (Table 3.10). Lag times were therefore greater in the sediment-containing microcosms than in the microcosms consisting of wetland materials. In the wetland microcosms, more mixing between the solid layer and the overlying liquids occurred, possibly because the mud was less dense than the sediment material and did not settle as tightly. Plant material also protruded from sediment layer into the aqueous layer in these microcosms. In addition, the east wetland microcosm was vigorously aerated after 15 d of incubation, because O_2 levels had dropped below detectable levels; this aeration caused complete mixing of the materials in the microcosm. Holm et al. (1992) determined that to a large extent, biological activity in a soil microcosm is associated with particulate matter rather than with the liquid portion. It is therefore possible that in the 2.5-L microcosms, insufficient mixing of the aquifer sediments or wetland materials with the aqueous portion of the microcosms may have resulted in reduced exposure of the microbial population to sulfolane, thereby decreasing the potential for biodegradation to occur.

By the end of the 377-d incubation, the east plume and west plume sediments in the viable microcosms had developed black regions, whereas the sediments in the corresponding sterile controls had remained a sandy-brown color throughout. This blackening of sediments was characteristic of the production of iron sulfides, which could have risen from the activity of $SO_4^{=}$-reducing bacteria in the sediment. The presence of $SO_4^{=}$-reducing bacteria in aquifer sediments from site 2 was demonstrated (Section 3.2.2), but there was no evidence from laboratory studies that sulfolane was degraded by $SO_4^{=}$-reducing bacteria (Section 3.3.4) during the 377-d incubation period. The sediment in the sterile east plume microcosm contained 1 µg/g sulfide (per wet weight sediment); no sulfide was detected in the sterile west plume microcosm. The east and west plume viable microcosms contained 510 µg/g and 760 µg/g sulfide (per wet weight sediment). This demonstrated that $SO_4^{=}$ reduction occurred in the settled aquifer sediments in the 2.5-L microcosms, which suggests that anoxic microsites were present in these microcosms, although the dissolved O_2 concentrations in the water layer remained high. $SO_4^{=}$-reducing bacteria are unlikely to be active in the presence of dissolved O_2 (Iverson and Olson, 1984; Jørgensen, 1983; McInerney et al., 1992; Postgate, 1979). Thus, both aerobic and anaerobic environments likely existed in the 2.5-L microcosms.

Kinetic analysis of the data for sulfolane loss in the viable microcosms showed similar rates (Table 3.10). In all cases, degradation followed zero-order kinetics. As it is unlikely that the bacterial populations in each microcosm were similar, particularly between the wetland materials and the aquifer sediments, it is reasonable to suggest that nutrient limitations existed in the aquifer sediment microcosms. The addition of P to the aquifer sediment microcosms allowed sulfolane degradation to proceed; at this point the limiting factor to degradation must have been similar to that observed in the wetland microcosms, as the rates of degradation were similar at this point. This suggested that O_2 might have been the limiting factor to biodegradation, once P had been provided. Assuming that O_2 levels were similar in all the microcosms and that biodegradation took place at the sediment:surface interface, the limitation in providing O_2 to the active microbial population would be the surface area of the settled sediment. It is also possible that mixing played a role in limiting sulfolane biodegradation. It was demonstrated that anaerobic sulfolane degradation plays a small role in removal of this compound from aquifer sediments at best (Section 3.2.4). It was also observed that the settled sediment portions of the 2.5-L microcosms had developed an active $SO_4^=$-reducing population over time (above) and were likely to be anaerobic. Holm et al. (1992) determined that the majority of a bacterial population in the environment is associated with fine sediment particles; interaction between bacteria attached to the sediment and the O_2-containing aqueous portion of each microcosm may have limited the rate of sulfolane biodegradation. This interaction would have been governed by mixing.

Nielsen et al. (1996) used laboratory microcosms similar to those of Holm et al. (1992) and to this study in order to determine the first-order biodegradation rate constants of several organic compounds. The concentration of dissolved O_2 remained constant at >9 mg/L in their work. Similar results were obtained for contaminated aquifer sediments for site 2. The wetland materials contained a great deal of organic C due to the presence of plant matter; decreases in the dissolved O_2 in these microcosms were likely due to utilization of these substrates. Nielsen et al. (1996) only examined aquifer sediments in their study; therefore it is not possible to refer to their work to support this hypothesis.

3.3.4 Respirometer microcosms

Salanitro et al. (1996; 1997) studied BTEX biodegradation in the presence of reduced levels of dissolved O_2. These authors used a custom-built, pressure transducer-controlled Comput-OX Respirometer (N-CON Systems Inc., Crawford, GA) to control O_2 concentration and monitor O_2 consumption in eight microcosms, simultaneously. Each microcosm consisted of 50 g of aquifer soil and 550 mL of groundwater in a 600-mL bottle modified to accommodate a dissolved O_2 probe, with a serum stopper port for amending and sampling the microcosms. An on-line computer monitored the volume of O_2 consumed. The achievable dissolved O_2 concentrations in the slurry ranged from 0.1 mg/L to 9 mg/L (Salanitro et al. 1996; 1997). With 5% O_2 in N_2 in the headspace, the dissolved O_2 in the slurry could be maintained near 2 mg/L. Changes in the input gas concentrations were used to maintain other dissolved O_2 levels (Salanitro et al., 1997). Their data suggested that microbial hydrocarbon metabolism can occur at dissolved O_2 concentrations of 0.2 to 2 mg/L, provided that the O_2 supply is constantly replenished. The net decay rates ranged from 0.3 to 0.7 mg/d when the dissolved O_2 ranged from 0.2 to 6.8 mg/L.

The ability of aquifer sediment microcosms to biodegrade sulfolane under conditions of reduced O_2 (approximately 2 mg/L) was tested using the respirometer described by Salanitro et al. (1996; 1997); this setup is described in Section 2.7.2.4. Sulfolane degradation at higher levels of dissolved O_2 (approximately 7.5 mg/L) was demonstrated in the 2.5-L microcosms (Section 3.3.3); in these microcosms O_2 replenishment during biodegradation was required, and the O_2 was replenished by slow mixing. Eight microcosms were monitored: four viable microcosms consisting of east plume sediment and groundwater, and two viable and two sterile control microcosms consisting of uncontaminated sediment and groundwater.

Sulfolane biodegradation was observed only in one viable microcosm containing east plume materials, and one containing uncontaminated materials (Figure 3.12). The lag time before degradation commenced was 90 d in the east plume microcosm and 102 d in the uncontaminated microcosm, and the rates of degradation were 0.28 mg/L/h and 0.32 mg/L/h, respectively. These biodegradation rates were comparable to those observed for the east plume 2.5-L microcosm, which was 0.5 mg/L/h (Section 3.3.3). Although dissolved O_2 was consumed in these microcosms during sulfolane biodegradation, it was consumed at a similar rate throughout the incubation period Figure 3.12), therefore this consumption could not be attributed to sulfolane

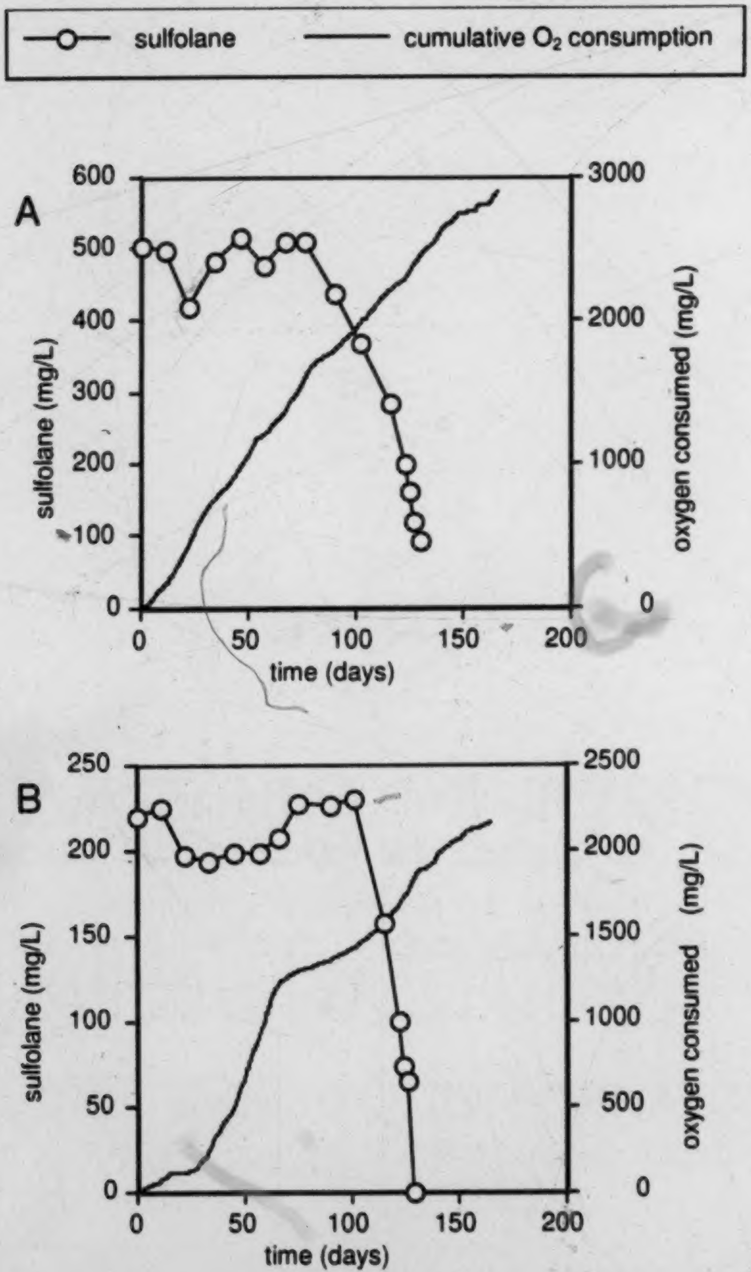


Figure 3.12. Sulfolane biodegradation and consumption of dissolved O_2 in respirometer microcosms inoculated with aquifer sediment and groundwater. Microcosms were incubated at 10°C. A. East plume sediment microcosm. B. Uncontaminated sediment microcosm supplemented with sulfolane.

biodegradation. The intention of this experiment was to demonstrate that sulfolane biodegradation was possible in aquifer sediments where a low level of dissolved O_2 was present. At the beginning of the experiment the dissolved O_2 concentration was 2.5 mg/L; after the 137-d incubation period dissolved O_2 concentrations were between 0.25 mg/L and 0.35 mg/L. These results demonstrated that sulfolane biodegradation could occur in the presence of low levels of dissolved O_2, but this activity was only demonstrated in one third of the viable microcosms.

3.3.5 Comparison of biodegradation results obtained from the different nutrient amendment and incubation conditions experiments

The experiments done to detect the sulfolane-degrading populations present at different locations at site 2 and the nutrient requirements of these populations encompassed three major parameters: the level of aeration, the extent of mixing, and the amounts of N and P supplementation. The most vigorous aeration and mixing were in the shake-flask cultures. The least amount of aeration was in the respirometer microcosms, however the contents of these microcosms were mixed during incubation. The least amount of mixing was in the 2.5-L microcosms, in which a layer of settled sediment or mud was covered by 10 to 15 cm of aqueous sample. The O_2 concentration in the aqueous layer of these microcosms was 7.5 to 9 mg/L, however the sediment portion of the microcosms was demonstrated to have anoxic sites, by the occurrence of $SO_4^{=}$ reduction, in two cases. The varied amounts of N and P supplementation were mainly tested in the shake-flask cultures; these demonstrated the highest biodegradation rates for sulfolane and were therefore the most suitable choice for large-scale experimentation. The concentrations of N and P used ranged from zero to concentrations that were in excess of the C:N:P ratio of 100:5:1 recommended by Alef and Nannipieri (1995).

It is possible to compare the biodegradation rates and lag times for sulfolane utilization by microbial populations from the different locations at site 2 (Table 3.11). Rates were considered to be similar if they differed by less than a factor of three, for the purposes of this comparison. The biodegradation rates in shake-flask cultures were higher than those in the 2.5-L microcosms in four of the eight sets compared: the uncontaminated sediment cultures and microcosm before and after the addition of N and P, and the east plume and west plume cultures and microcosms after the addition of P. The biodegradation rates for unsupplemented shake-flasks and microcosms consisting of materials from the east and west plumes and east and west wetland regions were

Table 3.11. Comparison of sulfolane biodegradation rates and lag times from shake-flask cultures, 2.5-L microcosms and respirometer microcosms.

N and/or P added	source of inoculum	culture or microcosm	lag time (d)	biodegradation rate (mg/L/h)
no	east plume	shake-flask	98	0.6
		2.5-L	225	0.5
		respirometer[a]	90	0.3
	west plume	shake-flask	34	0.4
		2.5-L	249	0.3
	uncontaminated	shake-flask	88	9.4
		2.5-L	>337[b]	0.0[b]
		respirometer[a]	102	0.3
	west wetland	shake-flask	9	0.2
		2.5-L	39	0.3
	east wetland	shake-flask	53	0.2
		2.5-L	25	0.4
yes	east plume	shake-flask	13	3.7
		2.5-L	4	0.5
	west plume	shake-flask	12	5.2
		2.5-L	28	0.3
	uncontaminated	shake-flask	24	1.7
		2.5-L	116[c]	0.0[c]

[a] One of four east plume replicate microcosms and one of two uncontaminated microcosms demonstrated sulfolane biodegradation in the respirometer experiment during the 140-d incubation period. The other replicates failed to degrade sulfolane over this time period, therefore displayed biodegradation rates of 0.0 mg/L/h.

[b] No biodegradation of sulfolane was observed during the 337-d incubation period. At 221 d, $1P_r$ was added to the microcosm. At 294 d, $1N_r$ and a second dose of $1P_r$ were added to the microcosm.

[c] No biodegradation of sulfolane was observed in this microcosm during the 116-d period after the addition of $1P_r$ to the microcosm, nor during the 41-d incubation after the addition of $1N_r$ and a second dose of $1P_r$.

fairly similar (Table 3.11). The active respirometer microcosm containing east plume aquifer materials showed a similar biodegradation rate to those of the 2.5-L microcosm and the shake flask culture. However, the active respirometer microcosm containing uncontaminated aquifer materials differed in that some biodegradation occurred, unlike in the 2.5-L microcosm, but the rate was substantially lower than that seen in the shake-flask culture. This set of results suggests that mixing was absolutely required for the development of a sulfolane-degrading population in the uncontaminated aquifer sediment and groundwater, and that mixing and aeration together were required to substantially improve the activity of this population. The mixing in the shake-flask culture was also much more vigorous than that seen in the respirometer microcosm. In contrast, active populations appeared to be present in the east and west plume aquifer sediments, and these populations appeared to require the addition of P to stimulate higher levels of activity, regardless of mixing. For the east plume aquifer sediment, the level of O_2 present also had little effect on the biodegradation rate (Table 3.11), provided that some O_2 was available.

After the addition of P to the east and west plume 2.5-L microcosms, the lag time was shorter for the east plume microcosm and longer for the west plume microcosm than for P-supplemented shake-flask cultures. These data were insufficient for any conclusions to be drawn regarding differences in lag times after nutrient supplementation between the types of experimental setup. Although biodegradation rates were similar in the unsupplemented microcosms and shake-flasks containing contaminated aquifer sediments or wetland materials, the lag times for three of the four sets were substantially longer in the microcosms than in the shake-flasks (Table 3.11).

Fu et al. (1996) studied phenol biodegradation in slurry, wafer and compacted soil tube reactors. Our shake-flask method was equivalent to their slurry reactor, and the 2.5-L microcosms were most similar to their compacted soil tube reactors. In the two experiments described by Fu et al. (1996), the lag times before phenol biodegradation were longer for the compacted soil tube reactor than for the slurry reactor, and the biodegradation rates for phenol (as measured by O_2 uptake) were substantially higher in the slurry reactor. These data agreed with the majority of the results for sulfolane removal from unsupplemented shake-flask cultures and microcosms.

Overall, it is likely that different factors affected sulfolane biodegradation, depending on whether the sediment or mud sample had been previously exposed to high concentrations of the compound. In samples with little or no previous exposure to sulfolane, the presence of a diverse microbial community, such as one would find in the wetland materials, appeared to be the most important factor, whereas in samples which had been previously exposed to the compound, nutrient limitation appeared to be the most significant factor governing biodegradation.

Supplementation of shake-flask cultures and microcosms containing aquifer sediments with P appeared to have the greatest influence on sulfolane biodegradation. The lag times for sulfolane biodegradation decreased markedly with the addition of P to the west plume and east plume materials (Table 3.9). In addition, Figure 3.10 and Table 3.7 show the strong correlation between the rate of sulfolane biodegradation and the concentration of P added to shake-flask cultures containing east plume materials. The availability of P in the microcosms therefore appeared to be the factor that limited sulfolane biodegradation most in the contaminated aquifer sediments. Further research is required to determine which factors most affect sulfolane degradation in the two wetland regions. From these data, it appeared that the microbial communities present in these regions were able to degrade sulfolane without aeration or the addition of N or P. Sulfolane levels in the wetlands were < 5 mg/L (Table 2.2), although the wetlands were then at the leading edge of a contaminant plume containing > 100 mg/L sulfolane. This suggested that *in situ* sulfolane biodegradation may have occurred in the wetlands at site 2, although the decrease in sulfolane concentration may have also been due to dilution by surface water.

In general, the minimum concentrations of N and P required for biodegradation at a given site will vary, depending on the nutrient requirements at the location. As well as varying between sites, nutrient requirements will vary at each site. The major effect will probably be due to differences in organic C concentrations.

In the case of site 2, the natural P concentration appeared to be lower than the amount of P required for sulfolane biodegradation in the east and west plume contaminated sediments and groundwaters. The natural N concentration did not appear to be unduly low for removal of these compounds from contaminated sediment and groundwater samples, given the evidence from this project. Gieg et al. (1999) showed that the aerobic biodegradation of DIPA liberates ammonium, which is a usable N source, thus once DIPA biodegradation begins in an aquifer, it will contribute to the amount of available N, and DIPA was a co-contaminant with sulfolane in these aquifer sediments. The minimum requirement for addition of N and P to the contaminated locations at site 2 could not be calculated from the information gained by this project, which was designed to determine whether N or P was more important for biodegradation and whether addition of either would stimulate biodegradation.

3.4 PAT medium for the detection of sulfolane-degrading organisms

Some of the information presented in Section 3.4 has been published previously by Greene and Fedorak (1998). This information is presented in this thesis with the full knowledge and permission of the co-author. PAT medium is described in Section 2.10.

Some difficulties arose in obtaining aerobic sulfolane-degrading bacterial isolates from contaminated sediments. Isolates obtained on various non-selective, rich media were rarely able to degrade the compound, and bacterial growth on minimal medium containing sulfolane as a sole C source was poor (unpublished results). A generalized indicator medium, tetrazolium agar, was tried with sulfolane as the C source. The ability of this medium to detect activity against a specific compound is based on the reduction of 2,3,5-triphenyl tetrazolium chloride to a deep red formazan during substrate oxidation (Bochner and Savageau, 1977). Preliminary work with tetrazolium salt agar containing sulfolane was unsatisfactory. The detection of a sulfolane degradation end product proved more useful for isolating sulfolane-degrading bacteria than the detection of a redox shift in the medium.

Two pH indicator-based media were designed by Loos (1975) to detect microorganisms that were able to degrade the pesticide 2,4-dichlorophenoxyacetic acid (2,4-D). This author used a bromocresol purple liquid medium in conjunction with an eosin-methylene blue agar to detect 2,4-D degraders. Yeast extract was provided as an alternate C source; the author calculated that the metabolites from bacterial utilization of yeast extract had a buffering effect (100 mg/L yeast extract was able to render the production of up to 0.24 mmol HCl undetectable with the pH indicators used). Mineralization of 1 mol 2,4-D resulted in the production of 1 mol HCl (Loos, 1975). Supplementation of the medium with 50 mg/L 2,4-D was therefore sufficient to overcome this buffering effect. The conclusion was that this medium would be useful for detecting microorganisms able to degrade 2,4-D and similar pesticides, provided that acid was released during mineralization.

Based on the media previously designed by Loos (1975) and Bochner and Savageau (1977), a medium to detect sulfolane-degrading bacteria was developed. A differential medium was postulated to be more useful than a selective medium, as it would allow the enumeration of total heterotrophic bacteria and sulfolane-degrading bacteria in a single sample, which could be useful for site monitoring experiments. The differential medium that was developed was called pH agar with tetrahydrothiophene-1,1-dioxide (PAT).

3.4.1 Development of PAT medium

Chou and Swatloski (1983) demonstrated a stoichiometric release of $SO_4^=$ during sulfolane biodegradation, and a corresponding decrease in the pH of the culture medium due to acid release concomitant with $SO_4^=$ production. The aerobic mineralization of sulfolane can be summarized by the equation shown in Table 3.5; 2 mol H^+ are produced during the degradation of 1 mol sulfolane. The production of acid from sulfolane biodegradation was used as the basis for developing a differential medium to detect sulfolane-degrading bacteria. Due to the previously observed poor growth of bacteria on agar with sulfolane as a sole C source, one-tenth strength Bacto Plate Count Agar (PCA) was used in the medium to encourage the development of easily visible colonies. Breakdown of the proteins present in PCA was predicted to produce basic ions, such as ammonia. The presence of sulfolane at concentrations as high as 1 g/L was not inhibitory to its biodegradation by aerobic mixed bacterial cultures, therefore this amount of sulfolane was added to the differential medium. Aerobic mineralization of 1 g of sulfolane was predicted to produce 16.7 mmol HCl, which would be easily detectable over the buffering effect of any protein catabolism byproducts in the medium.

Due to the potential toxicity of the EMB indicator dye used by Loos (1975), other pH indicator dyes were considered for the detection of acid produced by sulfolane biodegradation. Experiments with sulfolane-degrading cultures in unbuffered medium suggested that acid production commenced rapidly once sulfolane degradation began, and that the pH of the cultures would drop to pH 3.5 to 4.5 before bacterial activity was inhibited. The ideal pH indicator for sulfolane degradation therefore would demonstrate a color change between a pH of approximately 6.5 and pH 5.0. Bromthymol blue pH indicator (3,3'-dibromothymolsulfonphthalein) is yellow at pH $\leq$ 6.0, green at pH 6.0 to 7.6 and blue at pH $\geq$ 7.6. This indicator therefore had the advantage of being able to detect utilization of the protein portion of the medium, which would cause an increase in pH, as well as utilization of sulfolane, which would cause a decrease in pH. Bromocresol purple (Loos, 1975) did not offer the second type of detection, therefore this indicator was not tested. Bromthymol blue pH indicator was tested in liquid cultures of sulfolane-degrading mixed bacterial populations in order to demonstrate that it was not toxic to these bacteria; at concentrations of up to 200 mg/L this compound did not adversely affect sulfolane degradation.

The initial formulation of PAT medium was 2.4 g PCA, 0.1 g bromthymol blue, 1 g sulfolane and 15 g agar per 1 L deionized, distilled water. The addition of 2.4 g PCA brought the actual agar concentration in PAT medium to 16.5 g/L (1.65% w/v). PAT medium is initially dark green; bromthymol blue is green at pH 7. As heterotrophic bacterial colonies grew on PAT medium, they usually developed deep blue halos, presumably due to the release of ammonium ions or other alkaline metabolites during utilization of the tryptone in the one-tenth strength PCA. After the initial development of a blue color, sulfolane-degrading bacterial colonies developed a brilliant yellow halo, indicating the release of acidic products. The yellow halos are evident around the sulfolane-degrading colonies seen in Figure 3.13.

Unfortunately, the yellow color quickly diffused into the surrounding agar, making it difficult to determine which colonies were the sources of acid. Higher agar concentrations were tested to determine the optimal concentration for PAT medium. The results of this assay are shown in Table 3.12. The agar concentration did not appear to affect bacterial growth; plate count results were relatively consistent over the six agar concentrations tested (Table 3.12). Concentrations of agar above 2% prevented the development of a yellow color in sulfolane-degrading colonies, although a sulfolane-degrading population was present and was detected on plates with lower agar concentrations after 14 d incubation. The yellow halo around each positive colony spread to 2 mm shortly after it appeared on the 1.65% agar plate, whereas yellow halos detected on the 1.75% and 1.85% agar plates spread only 1 mm. No halo was detectable on the 1.95% agar plates, although positive colonies turned yellow. The appropriate agar concentration should allow the development of a yellow halo around positive colonies, without allowing it to spread too far, therefore 1.85% (w/v) was deemed to be the optimum agar concentration for PAT medium.

The data in Table 3.12 demonstrate a common problem with the enumeration of sulfolane-degrading bacteria. Sulfolane-degrading populations were often found to be below the recommended number of 30 to 300 colonies on plates where the number of total heterotrophic bacteria was countable. However, incubation of PAT medium plates with a lawn of heterotrophic bacteria for an additional 2 to 4 wk frequently allowed the development of discrete, sulfolane-degrading colonies that were easily counted on top of the lawn of indistinguishable non-degrading organisms. These bacterial colonies could be transferred to fresh medium, isolated, and demonstrated to degrade sulfolane in liquid medium, therefore this counting technique, although unconventional, proved to be

Figure 3.13. Sulfolane-degrading bacterial colonies on PAT medium plates. Sulfolane-degrading colonies are indicated by the light halo around bacterial growth.

Table 3.12. The effect of different agar concentrations in PAT medium on bacterial growth and the detection of sulfolane-degrading bacteria. Plates were spread with 0.1 mL of a 10^{-5} dilution of a 200-mL shake-flask culture inoculated with 20 mL of a culture consisting of 200 mL groundwater + 50 g sediment from the contaminated west plume aquifer at site 2. Cell counts are the result of averaging three replicate plate counts.

agar concentration (%)	total heterotrophs in dilution (CFU/mL)	sulfolane-degraders in dilution (CFU/mL)	sulfolane-degrading colony diameters (mm)	yellow halo diameters (mm)
1.65	165	4	0.5-8.0	2.0
1.75	159	3	0.5-10.0	0-1.0
1.85	174	3	0.5-3.0	0-1.0
1.95	169	1	0.5-5.0	0
2.05	166	0	1.0-5.0[a]	NM[b]
2.15	175	0	1.0-4.0[a]	NM[b]

[a] Colonies suspected of the ability to degrade sulfolane (due to their similarity in colony morphology to known sulfolane degraders) were measured. These colonies were streaked on PAT medium with a lower agar content to confirm their sulfolane-degrading abilities.

[b] NM not measured. No yellow colonies were observed therefore no halos were present to be measured.

successful in most cases. Occasionally, sulfolane-degraders could not be distinguished in this manner; these plates were deemed uncountable and sulfolane-degrading populations were not detected in these cases.

3.4.2 Comparison between PAT medium and LB agar for enumerating total heterotrophs

Loos (1975) observed that the methylene blue dye present in agar for the detection of 2,4-D-degrading bacteria could be inhibitory to some organisms. PAT medium contained 1 g/L sulfolane and a pH indicator dye, bromthymol blue. Neither of these compounds was toxic to a mixed, sulfolane-degrading population (data not shown), however they might have proved to be inhibitory to some environmental microorganisms. Thus, a comparison between a standard rich medium (LB agar) and PAT medium was made. The microbial populations of seven different soils were evaluated using PAT medium and LB agar. For each soil, at the dilution which yielded the most accurate colony counts, 10 plates of each agar type were enumerated (Table 3.13). For six soils and sediments, PAT medium counts ranged from 2% to 47% of the counts obtained on LB agar. However, for the site 2 west plume soil the PAT medium count was 126% of the LB agar count. The use of PAT medium also resulted in a decrease in the accuracy of plate-count assays. The standard deviations for these plate counts (Table 3.13).

In general, fewer microorganisms grew on PAT medium than on LB agar. The decreased level of growth on this medium was likely due to two things. First, sulfolane, or bromthymol blue, may have been toxic to some of the microorganisms. Second, it was possible that the reduced amount of nutrient sources in PAT medium decreased the number of total heterotrophic colonies able to develop when compared with a full-strength rich medium. Plate count enumeration is not the best method for detecting all the microorganisms in an environmental sample. Buck (1979) stated that many factors can affect the fraction of total viable microorganisms in a given environmental sample are likely to be cultureable on agar plates. Microorganisms can form aggregates with soil, thus decreasing the total number of CFU observed. In some cases, cells may not be capable of growing on the given nutrient agar (Buck, 1979). Thus, the difference between enumeration on LB agar and PAT medium, which was less than 10-fold in most cases (Table 3.13), was not likely to have a strong effect on the overall enumeration of total cultureable heterotrophic bacteria in each sample. The important reason for using

Table 3.13. Colony counts for 7 soils on PAT medium and LB agar. The CFU/g soil were obtained by averaging the results from 10 plate counts at a single dilution. The soils used in this experiment are described in Tables 2.2 to 2.4.

soil identity	LB agar		PAT medium		PAT to LB count ratio (%)	t value[a]
	average CFU/g soil	standard deviation	average CFU/g soil	standard deviation		
site 2 west plume	8.68×10^6	1.27×10^6	1.10×10^7	2.25×10^6	126	2.694
site 2 Sulfinol® #1	1.73×10^6	1.28×10^6	2.64×10^5	4.12×10^4	15	3.434
site 2 Sulfinol® #2	1.63×10^7	1.30×10^6	4.04×10^5	1.12×10^5	2	36.5
topsoil	4.16×10^7	9.08×10^6	1.70×10^7	4.31×10^6	41	7.332
greenhouse soil	4.65×10^6	9.10×10^5	2.16×10^6	3.31×10^5	47	7.717
humus rich soil	9.21×10^5	1.08×10^5	3.25×10^5	6.45×10^4	35	14.2
uncontaminated	9.34×10^6	1.06×10^6	3.68×10^6	1.28×10^6	39	10.22

[a] A total of 20 plate counts (10 on PAT medium, 10 on LB agar) were done for each soil, therefore the degrees of freedom were $df = n_1 + n_2 - 2 = 18$. For 18 degrees of freedom, and $P = 0.05$, $t_{crit} = 2.093$. For all the samples shown here, the t_{calc} was greater than the t_{crit}, therefore there was a significant difference between plate counts on PAT medium and on LB agar for all seven soil and sediment samples tested.

PAT medium would be to detect overall changes in the bacterial population, particularly with respect to the sulfolane-degrading population.

3.4.3 Detection of sulfolane-degrading bacteria on PAT medium

3.4.3.1 Growth of known bacterial strains on PAT medium

PAT medium was tested with 46 bacterial strains, including common laboratory types and environmental isolates (Table 3.14, sources summarized in Table 2.5). Each strain was grown in rich medium, then streaked onto PAT medium for testing and onto LB agar to ensure the purity and viability of the strain. In all cases, only sulfolane-degrading bacterial colonies demonstrated the development of a blue halo during initial colony growth, followed by the formation of a yellow halo.

For six of the bacterial species, the initial plate color was yellow, followed by the eventual development of a blue halo around the colonies (Table 3.14). This yellow color often appeared on the plate as the culture was streaked; it did not require any bacterial growth on PAT medium to develop. Once the colonies began to grow, a blue color developed on the PAT plates and the yellow halo did not reappear. A possible explanation for this phenomenon was that the culture used to inoculate the plates contained acidic products of bacterial growth. These isolates, *Paenibacillus polymyxa, Bacillus subtilis* ssp. niger, *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus faecalis* and strain LMII, all of which showed an initial yellow halo on PAT medium, were tested for their ability to degrade sulfolane in liquid cultures. It was found that none of the isolates was able to use sulfolane as a growth substrate. It was therefore important to monitor the development of bacterial colonies on PAT medium over a period of at least 10 d in order to discern which colonies really consisted of sulfolane-degraders, and which produced temporary false positive results.

The E. coli K12 NAR11 mutant tested on PAT medium was reported to oxidize sulfolane (Juhl and Clark, 1990); this ability was demonstrated by testing the organism on tetrazolium redox indicator plates (Abdulrashid and Clark, 1987), as described by Bochner and Savageau (1977). This organism grew on PAT medium, but did not produce a yellow halo, indicating that little or no acid was produced from sulfolane degradation. The *E. coli* strains provided by D. Clark were mutated to improve their ability to degrade thiophene (Clark et al., 1988). The authors demonstrated that no release of S occurred when the *E. coli* strains active against thiophene were grown in the presence of sulfolane or other S compounds, nor were the bacteria able to utilize these compounds as a source of S (Juhl and Clark, 1990).

Table 3.14. The detection of sulfolane-degrading bacteria on PAT medium using known bacterial strains and environmental isolates.

identification of bacterial isolate	color of halo (4 d)	color of halo (10 d)	sulfolane-degrader (Y/N)
Alcaligenes denitrificans	blue	blue	N
Bacillus cereus	NG[a]	blue	N
Bacillus polymyxa	yellow	blue	N
Bacillus subtilis ssp. niger	yellow	blue	N
Escherichia coli B15	blue	blue	N
Escherichia coli ESS	NG[a]	NG[a]	N
Escherichia coli K12 W1485	blue	blue	N
Escherichia coli K12 NAR11	blue	blue	N
Micrococcus luteus	NG[a]	NG[a]	N
Nocardia sp.	NG[a]	blue	N
Proteus vulgaris	blue	blue	N
Pseudomonas aeruginosa	blue	blue	N
Pseudomonas alcaligenes	blue	blue	N
Pseudomonas fluorescens	blue	blue	N
Pseudomonas putida	blue	blue	N
Pseudomonas putida	NG[a]	blue	N
Pseudomonas putida	NG[a]	blue	N
Pseudomonas putida	blue	blue	N
Pseudomonas putida	blue	blue	N
Pseudomonas putida	blue	blue	N
Pseudomonas putida	blue	blue	N
Pseudomonas putida	blue	blue	N
Pseudomonas sp. HL7b	NG[a]	NG[a]	N
Pseudomonas sp.	blue	blue	N
Rhodococcus sp.	blue	blue	N
Staphylococcus aureus	yellow	blue	N
Staphylococcus epidermidis	yellow	blue	N
Streptococcus faecalis	yellow	blue	N
Streptococcus pyogenes	NG[a]	NG[a]	N
Streptomyces clavuligerus	blue	blue	N

Table 3.14 continued.

identification of bacterial isolate	color of halo (4 d)	color of halo (10 d)	sulfolane-degrader (Y/N)
5R	blue	blue	N
DFC49	blue	blue	N
DFC50	blue	blue	N
HL4	blue	blue	N
LMI	blue	blue	N
LMII	yellow	blue	N
RJGII-135	blue	blue	N
HP1-S28[b]	blue	yellow	Y
HP5-S8[b]	blue	yellow	Y
HP8-S28[b]	blue	yellow	Y
HP81-S28[b]	blue	yellow	Y
WP1-S28[b]	blue	yellow	Y
WPS1-S28[b]	blue	yellow	Y
WPS2-S28[b]	blue	yellow	Y
WPU-S28[b]	blue	yellow	Y
WPW-S28[b]	blue	yellow	Y

[a] NG, no growth

[b] Isolate from contaminated sediment that utilized sulfolane as a sole source of C and energy.

The thiophene-degrading *E. coli* isolate was also tested in liquid medium containing sulfolane as a sole C source, or sulfolane as an alternate C source in the presence of either glucose or acetate, and it was not able to remove sulfolane from the medium under any of these conditions. It is possible that *E. coli* K12 NAR11 was able to modify sulfolane in some manner that affected the reduction of the tetrazolium indicator dye, but was unable to utilize sulfolane as a sole C and energy source, or to degrade the compound to the extent required for acid release. The importance of an indicator medium that provides positive results only upon the partial or complete destruction of the test compound, in this case sulfolane, was demonstrated by the failure of the tetrazolium redox indicator plates to distinguish between organisms that may have modified sulfolane slightly and those which were able to utilize sulfolane as a C source.

3.4.3.2 Limitations of PAT medium for enumerating sulfolane-degrading bacteria

Sulfolane-degrading bacteria were present at significantly lower numbers than the total heterotrophic population, even in sulfolane-contaminated soils. Uncontaminated aquifer sediment from site 2 or greenhouse soil was mixed with various amounts of a pure, sulfolane-degrading culture (WPW-S28, described in Section 3.7.2.2) to determine the proportion of sulfolane-degrading bacteria required for detection in a heterotrophic population on PAT differential plates. The number of sulfolane-degrading bacteria in both of the soils was initially <10 CFU/g soil (wet weight). In the uncontaminated sediment, the average heterotrophic count was 8.4 x 10^4 CFU/g soil. Within this background, WPW-S28 colonies were detectable at numbers as low as 100 CFU/g soil. (0.12% of the total heterotrophic count). In the greenhouse soil, sulfolane-degrading bacteria were detectable at 5.6 x 10^5 CFU/g soil in a background of 1.9 x 10^7 CFU/g soil (2.9% of the total heterotrophic count).

PAT medium was also used to enumerate heterotrophic bacteria and sulfolane-degrading bacteria in five samples taken from the site 2 sour gas treatment facility. The soil types and sulfolane concentrations present in the corresponding groundwater or surface water samples are summarized in Table 2.2. The total cultureable heterotrophic microbial population and the number of sulfolane-degrading bacteria present in each sample were determined (Table 3.15). For each given location, the number of sulfolane-degrading bacteria was at least 100-fold lower than the number of heterotrophic bacteria present. In two of the samples, sulfolane-degrading bacteria were not detected. The lowest detectable levels of sulfolane-degrading bacteria in the soils spiked with WPW-S28 were therefore similar to the microbial populations actually existing in contaminated wetlands and aquifer sediments, demonstrating that PAT medium was useful for detecting sulfolane-degrading populations at levels in which they exist in the field.

Experience showed that when the proportion of sulfolane degraders in a sample was small, incubation times of as little as 2 to 4 d were sufficient for sulfolane-degrading colonies to be distinguished. In some cases, long incubation times resulted in overgrowth of the plates by the total heterotrophic bacterial population. In many other cases, large sulfolane-degrading bacterial colonies eventually grew on top of the lawn of heterotrophic organisms. PAT medium is not selective for sulfolane degraders, hence the ability to detect small numbers of these bacteria in a large background of other heterotrophs is limited, however detection of sulfolane-degrading bacteria representing 0.1% of the heterotrophic population was possible. The most important factor for accurate enumeration of sulfolane-degrading bacteria was regular monitoring of PAT plates during incubation.

3.4.3.3 Enumeration of sulfolane-degrading bacteria in environmental samples

The relative enrichment of sulfolane-degrading bacteria in shake-flask cultures inoculated with various soil samples was monitored using PAT medium. Shake-flask cultures consisting of soil or wetlands material from each of the five site 2 sampling locations (Table 2.2) and B+N8P medium were given 30 mg sulfolane. Total heterotrophs and sulfolane-degrading bacteria were enumerated using PAT medium. After the removal of sulfolane, cultures were refed; after removal of the second dose of sulfolane, 10 mL of each culture was transferred to fresh medium containing sulfolane. The total heterotrophic and sulfolane-degrading bacterial counts obtained during the removal of sulfolane in the east plume transfer culture are shown in Figure 3.14. The numbers of sulfolane-degrading bacteria and heterotrophs both increased as sulfolane was consumed. By the end of the 150-h incubation, the number of sulfolane degraders was essentially the same as the number of total heterotrophs, demonstrating that sulfolane-degrading organisms were preferentially enriched when sulfolane was provided as the sole C and energy source. Similar results were seen for the other cultures that were studied. Figures showing the bacterial cell numbers during sulfolane removal for all the cultures are shown in Figure C1, in Appendix C. These data showed that the PAT medium could be used to monitor changes in the proportions of sulfolane-degrading bacteria with respect to the number of total heterotrophs in a microbiological treatment process designed to remove sulfolane.

Table 3.15. Total heterotrophic and sulfolane-degrading bacterial populations in samples taken from five locations at site 2. Bacterial populations were enumerated on PAT medium.

sample location	heterotrophic bacteria (CFU/g soil)	sulfolane-degrading bacteria (CFU/g soil)	% sulfolane degraders in the total heterotrophic population
east plume	3.2×10^6	1.2×10^4	0.38
west plume	7.2×10^6	3.9×10^4	0.54
uncontaminated	9.6×10^6	$< 4.0 \times 10^4$	< 0.42
west wetland	1.6×10^6	$< 4.0 \times 10^4$	< 2.5
east wetland	2.6×10^6	8.0×10^4	3.1

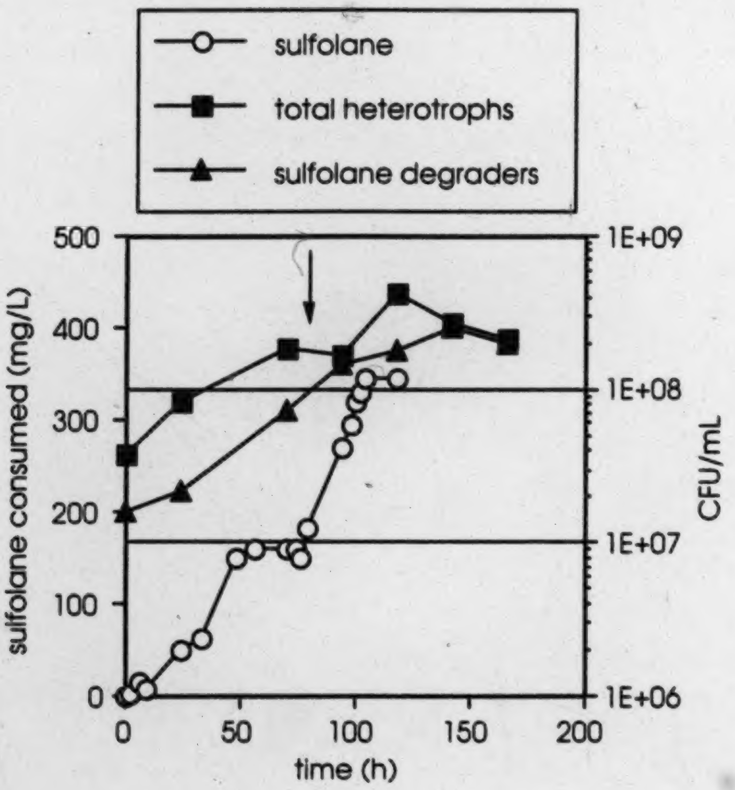


Figure 3.14. Changes in microbial counts in the site 2 east plume transfer culture as a result of sulfolane biodegradation at 10°C, determined by bacterial colony counts on PAT medium plates. The culture contained 10 mL sediment slurry culture and 200 mL highly buffered (B+N8P) medium, and was supplemented with 30 mg sulfolane at time zero and at 80 h (marked by the arrow).

3.4.4 Accuracy of PAT medium in predicting the ability of bacteria to degrade sulfolane

It was necessary to define the accuracy with which PAT medium could be used to predict the ability of individual bacterial colonies to utilize sulfolane as a sole C source. After incubation for 3 to 10 d, colonies were selected from plates of PAT medium that had been inoculated with the aquifer sediment and swamp samples, (Section 3.4.3.3), and used to determine the reliability of the differential medium. Individual colonies were used to inoculate test tubes containing medium supplemented with sulfolane and bromthymol blue pH indicator. Luria-Bertani agar was used to verify that the bacteria survived transfer into the liquid medium. In total, 144 positive colonies showing yellow halos were inoculated into sulfolane liquid medium, of which 135 were viable. Similarly, 136 negative colonies, showing no yellow halos, were transferred into sulfolane liquid medium; all were viable.

After 10 d of incubation, 10 of the negative isolates turned the sulfolane liquid medium yellow, whereas 126 of the positive isolates turned the sulfolane liquid medium yellow. The consumption of sulfolane in the liquid cultures that had turned yellow had caused the pH to drop to between 3.0 and 3.5, which limited the amount of sulfolane that was consumed. The pH of selected samples (approximately 10% of the total yellow tubes observed) was tested in order to determine that the change in medium color was initiated by a drop in pH. Each yellow culture was neutralized and incubated for another 10 d, then the sulfolane concentration was measured. Overall, 24 sterile control analyses were done, giving an average sulfolane concentration ± two standard deviations of 590 ± 44 mg/L. Sulfolane biodegradation was considered to have occurred when the measured concentration in a culture was less than the mean control value less two standard deviations (546 mg/L).

Using this criterion, of the 135 positive colonies picked from the PAT plates, 93% degraded sulfolane in liquid medium. Interestingly, two of the active cultures were able to utilize bromthymol blue pH indicator as well as sulfolane; both these culture tubes became colorless over the incubation time. Catabolism of the bromthymol blue dye might have resulted in a false positive result on PAT agar, should these organisms have been unable to degrade sulfolane as well as bromthymol blue. This hypothesis may explain the advent of 'false positive' colonies on PAT medium, however it was not tested. Of the 136 negative colonies, 93% of them did not degrade sulfolane. Colonies that were able to degrade sulfolane but appeared negative on PAT medium might have been less active than some positive colonies, causing insufficient acid production for the development of a yellow halo on PAT medium.

This experiment demonstrated that PAT medium could be used to successfully isolate and enumerate sulfolane-degrading bacteria from environmental samples. Because PAT is a differential medium, the same plates could be used to estimate the total number of cultureable heterotrophic bacteria, as well as the number of sulfolane-degrading bacteria in a sample.

3.5 A model system designed to evaluate an air-sparged aquifer in the field

This series of experiments was designed with two purposes in mind: first, to determine the effects of nutrient amendment on sulfolane biodegradation in an aerated, solid phase system, and second, to assess the effects of this amendment on the total heterotrophic and sulfolane-degrading bacterial populations. A third purpose was added to this plan: to determine the possibility of predicting sulfolane biodegradation in the solid phase system using laboratory shake-flask cultures. This purpose was added because biodegradation in the solid phase system was predicted to take longer than it would in shake-flask cultures; as previously discussed (Section 3.3), shake-flask cultures present a simple, quick, easily replicated system that requires fewer materials than more complex, aquifer-like laboratory microcosms. Although shake-flask systems will likely overestimate biological activity at a site, they may be useful for assessing the potential for bioremediation of contaminated sites.

The experimental design for the air-sparged microcosms was based on the work of Mueller et al. (1991). This system has been used previously to examine sulfolane biodegradation (Fedorak and Coy, 1996), however these authors were unable to sample the chambers frequently due to the difficulties presented by sulfolane analyses. At the time, the method for analyzing sulfolane involved extensive Soxhlet extractions to remove sulfolane from the soil, which required realtively large samples, preventing frequent sampling. An aqueous sulfolane analytical method was developed during this thesis work. Removal of sulfolane from soils by water extraction was found to be efficient (Section 3.5.1), relatively simple, and could be carried out on small quantities of soil (1 g per extraction), which allowed much more frequent sampling of the air-sparged microcosms during this experiment.

3.5.1 Development of a system for aqueous extraction of sulfolane from soils

Luther et al. (1998) concluded that sulfolane was unlikely to interact with soil organic matter and that very little interaction between sulfolane and the clay mineral component of soils occurs. Sulfolane is soluble in water up to concentrations above 300 g/L (Merck Index, 1968). The solubility of sulfolane and its low affinity for soil components suggested it could be extracted from soil with water. Three matrices were used to determine the recovery rate of sulfolane from soil: a clean sand of 250-1000 μm diameter, a greenhouse soil high in organic matter and an uncontaminated aquifer sediment sample from the site 2 sour gas treatment plant (Tables 2.2 and 2.3).

Sifted, air-dried soil samples were loaded with sulfolane in acetone (Section 2.7.3.2). For each soil type, triplicate samples of soil were extracted with water. The percent recovery of sulfolane from the three soil types was 102% for the sand, 82% for the greenhouse soil and 104% for the uncontaminated aquifer sediment, suggesting that sulfolane could be recovered from soil samples by water extraction.

Further experiments were done with the site 2 uncontaminated aquifer sediment to determine the best conditions for recovery of sulfolane from soil. Samples of 1, 2 or 3 g were used (Table 3.16). It was demonstrated that 1 g samples yielded the best results for sulfolane extraction, and that leaving the soil at room temperature for up to 48 h prior to extraction did not cause a decrease in the amount of sulfolane that was recovered. For 1 g soil samples, two extractions appeared to be sufficient for sulfolane recovery. Each extraction took approximately 15 min.

3.5.2 Shake-flask cultures

Shake-flask cultures were designed to test the ability of the microbial populations of the different soils used to inoculate the air-sparged microcosms to degrade sulfolane. These cultures were therefore amended with the same amounts of N and P that were proposed for amendment of the air-sparged microcosm soils (Section 2.7.3.1). For Sulfinol®-contaminated soils, the amount of N and P amendment was calculated based on the total amount of sulfolane present in the soil inocula. Topsoil was used as a model soil because it had a large, diverse microbial population and high levels of organic matter, and had not previously been exposed to sulfolane. Sulfinol®-contaminated sediment was used to study a system that had previously been exposed to sulfolane.

3.5.2.1 Topsoil shake-flask cultures

The topsoil was 23% C, 1.3% N and 0.13% P. The C:N:P ratio of 100:5:1 would have been 1.15% N and 0.23% P, suggesting that P supplementation was required. The topsoil was modified with 0.8 mg sulfolane per 1 g soil (wet weight), which represented an additional 0.32 mg/g C in the soil. Given the C already present in the soil, 230 mg/g, the C from sulfolane represented only 0.1% w/w of the total C in the system. Nutrient

Table 3.16. Recovery of sulfolane from site 2 uncontaminated aquifer sediment samples. Soil was air dried, then loaded with sulfolane at a concentration of 0.1 mg/g soil dry weight. Each extraction was done in 5 mL water; each sample was extracted with a total of 15 mL water. The amount of time allowed to elapse between the addition of sulfolane to each sample and the beginning of the extraction procedure was 24 or 48 h. Each extraction took approximately 15 min to complete.

time elapsed before extraction	amount of soil extracted (g)	extraction number	sulfolane recovered (mg/g soil)	% of added sulfolane recovered	total % recovery of sulfolane
24 h	1	1	0.065	65	
		2	0.025	25	
		3	0	0	90
	2	1	0.055	55	
		2	0.017	17	
		3	0	0	71
	3	1	0.051	51	
		2	0.014	14	
		3	0.009	9	74
48 h	1	1	0.067	67	
		2	0.035	35	
		3	0	0	102
	2	1	0.056	56	
		2	0.021	21	
		3	0.012	12	89
	3	1	0.048	84	
		2	0.026	26	
		3	0.008	8	82

amendment was unlikely to have an effect on the lag time before commencement of sulfolane biodegradation in this soil, or on the biodegradation rate.

Two sets of nutrient concentrations were prepared: one supplemented with sufficient N and P to account for the C present in sulfolane only (designated 'low N and P'), and one supplemented with sufficient N and P to account for the total C present in the samples (designated 'high N and P'). Low levels of N addition stimulated sulfolane biodegradation; the lag time before sulfolane removal was halved from 6 d for unsupplemented or low P-supplemented cultures to 3 d for low N-supplemented or low-N and P-supplemented cultures (Figure 3.15, Table 3.17). Interestingly, supplementation of the cultures with higher levels of nutrients resulted in longer lag times before the commencement of biodegradation; cultures that were supplemented with high P or high N and P demonstrated a lag time of 7 d, while the culture supplemented with high N had a lag time of 24 d (Figure 3.15, Table 3.17). No net loss of sulfolane from the sterile control was observed over the incubation time (Figure 3.15). It is possible that the addition of higher levels of nutrients to the cultures stimulated the biodegradation of organic C compounds other than sulfolane; this was not investigated. It is unlikely that the cultures were inhibited by an excess of N; this possibility was discussed by Braddock et al. (1997), however these authors felt that inhibition by N was caused by the presence of NH_4^+. In this experiment N was added in the form of NO_3^-, therefore NH_4^+-mediated inhibition of biodegradation did not occur.

3.5.2.2 Site 2 contaminated sediment shake-flask cultures

The nutrient requirements for biodegradation of sulfolane in the site 2 contaminated sediment were not known, nor was it known whether this soil contained any compounds that might prove inhibitory to sulfolane biodegradation. The total C in this sediment was 2.23%, or 22.3 mg/g soil. The sulfolane concentration was 7.8 mg/g soil, which accounted for only 14% of the total C present. The same nutrient concentrations were tested as for the topsoil shake-flask cultures (Section 3.5.2.1). Shake flask cultures supplemented with trace minerals solution or B+N8P medium were also incubated. One shake flask culture was inoculated with 10 g contaminated sediment rather than 50 g sediment in case products toxic to sulfolane-degrading microorganisms existed in the soil.

Shake-flask cultures supplemented with low N or low N and P demonstrated a lag time of 27 d, and a sulfolane degradation rate of 0.6 and 0.4 mg/L/h, respectively. The unsupplemented and low P-supplemented cultures both had a lag time

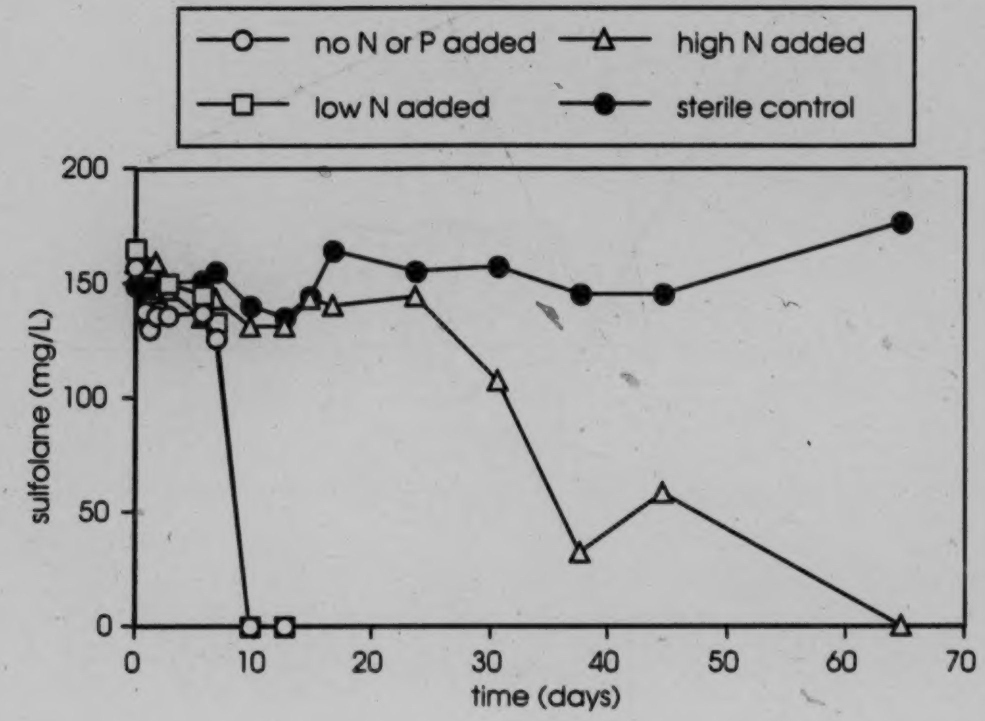


Figure 3.15. Removal of sulfolane from shake-flask cultures inoculated with topsoil. Cultures were incubated at room temperature. Low N or P refers to sufficient N or P to account for the sulfolane-C in a sample, according to the C:N:P ratio of 100:5:1. High N or P refers to sufficient N or P to account for the total C in a sample.

Table 3.17. Removal of sulfolane from shake-flask cultures inoculated with topsoil. Low N or P refers to sufficient N or P to account for the sulfolane-C in a sample, according to the C:N:P ratio of 100:5:1. High N or P refers to sufficient N or P to account for the total C in a sample. Samples were incubated at room temperature.

sample description and supplementation	lag time (d)	biodegradation rate (mg/L/h)[a]
no N or P	6	1.5
low N	3	1.5
low P	6	1.6
low N + P	3	1.8
high N	24	0.14[b]
high P	7	2.3
high N + P	7	2.1

[a] The biodegradation rate was calculated using only 3 data points as biodegradation of sulfolane occurred so quickly in these cultures once the lag time was over.

[b] The biodegradation rate was calculated using 5 data points; in this sample microbial activity against sulfolane was much slower than in any of the other samples.

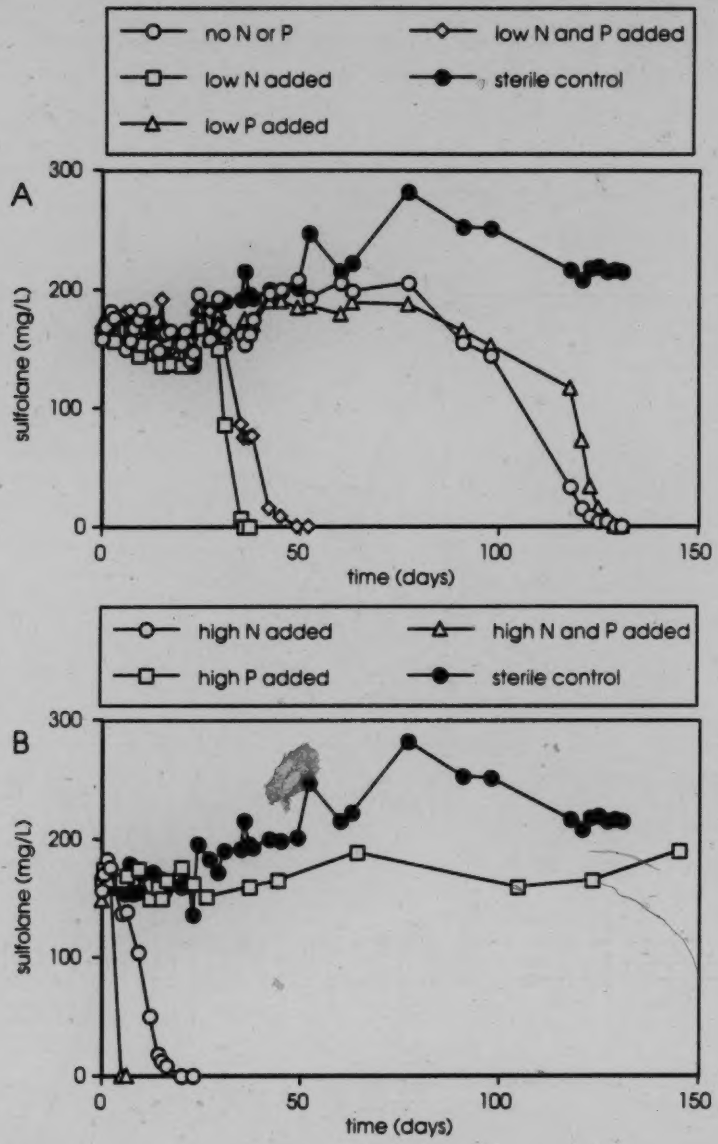


Figure 3.16. Removal of sulfolane from shake-flask cultures inoculated with aquifer sediment taken from near the Sulfinol Building #2 at site 2. Cultures were incubated at room temperature. Low N or P refers to sufficient N or P to account for the sulfolane-C in a sample, according to the C:N:P ratio of 100:5:1. High N or P refers to sufficient N or P to account for the total C in a sample. Soil inocula were 50 g except in the 'low soil' culture; which contained 10 g soil. A. Cultures supplemented with low levels of N or P. B. Cultures supplemented with high levels of N or P.

Table 3.18. Removal of sulfolane from shake-flask cultures inoculated with sediment sampled from Sulfinol® Building #2 at site 2. Low N or P refers to sufficient N or P to account for the sulfolane-C in a sample, according to the C:N:P ratio of 100:5:1. High N or P refers to sufficient N or P to account for the total C in a sample. Soil inocula were 50 g except in the culture labeled 'low soil'; this culture contained only 10 g soil inoculum. Cultures were incubated at room temperature.

sample description and supplementation	lag time (d)	biodegradation rate (mg/L/h)
no N or P	77	0.19
low N	27	0.64
low P	77	0.15
low N + P	27	0.44
high N	2	0.58
high P	>145[a]	0.0[a]
high N + P	2	1.9
trace elements	50	0.10
B+N8P medium	1	1.2
low soil inoculum	>145[a]	0.0[a]

[a] No biodegradation of sulfolane was observed in this culture.

of 77 d, with degradation rates of 0.2 and 0.1 mg/L/h, respectively (Figure 3.16A, Table 3.18). After a lag time of 2 d, sulfolane was completely removed from triplicate shake-flask cultures supplemented with the high N and P concentrations by 5 d (Figure 3.16B) at a rate of 2 mg/L/h. Removal of this compound from cultures supplemented with high levels of N was observed over a period of 14-16 d (Figure 3.16B) at a rate of 0.6 mg/L/h, after a lag time of 2 d. No sulfolane removal was observed in cultures supplemented with high levels of P over an incubation period of 145 d (Table 3.18). The sulfolane concentration in this shake-flask culture appeared to increase over time, as it did in the sterile control culture (Figure 3.16B). These cultures were sealed with a foam plug, therefore the increase in sulfolane concentration observed was probably the result of concentration of the compound due to evaporation of water from the culture flasks. The lag time for sulfolane degradation in B+N8P medium was 1 d, and the rate or removal was 1.2 mg/L/h, while for the culture modified with trace mineral elements the lag time was 50 d and the degradation rate 0.1 mg/L/h, demonstrating that trace nutrients were not lacking in these cultures. No biodegradation was observed over an incubation period of 145 d in the culture inoculated with only 10 g soil (Table 3.18).

Overall, P supplementation was of little importance for sulfolane removal from the Sulfinol® #2 sediment cultures unless sufficient of N was present (Table 3.18), whereas N supplementation was required to stimulate degradation of this compound. Removal of sulfolane was possible when no N was added. However, the addition of low N increased the ability of cultures to degrade sulfolane, and the addition of sufficient N to give a C:N:P ratio of 100:5:1 for the total organic C in the soil resulted in a much shorter lag time before sulfolane degradation commenced (Table 3.18). The aquifer sediment from this sampling location was 0.077% total N and 0.044% total P, representing 0.77 mg/g N and 0.44 mg/g P in the soil. Assuming that all the N and P detected in this soil was in a biologically available form, approximately half the required nutrients were available before the soil was amended. In spite of the shortage of N and P demonstrated by these calculations, sulfolane was removed from the unsupplemented culture. There were probably C-containing compounds in this soil that were not degraded; these must have been more recalcitrant to microbial degradation than sulfolane. These data suggested that biodegradation of sulfolane in the air-sparged microcosm experiment was likely to be stimulated by the addition of N, and that the addition of sufficient N to account for all the organic C present in the aquifer sediment might decrease the lag time before sulfolane removal began, when compared with addition of only sufficient N to account for the C provided by sulfolane.

3.5.3 Air-sparged microcosms

Air-sparged microcosms were similar to a biopile remediation setup in the field (Blackburn and Hafker, 1993). Mueller et al. (1991) described a 'landfarming chamber microcosm' experimental setup for predicting microbial activity in the field. Fedorak and Coy (1996) used a modification of this method to examine sulfolane degradation in contaminated sediments. This modified system (Section 2.7.3.2) was employed to further study sulfolane biodegradation. Although the initial work by Mueller et al. (1991) emulated landfarming treatment, this suggested a relatively thin layer of soil or sediment in the microcosms, and air exposure from the top. The system used for these experiments was more accurately described as a 'biopile' type of remediation (Blackburn and Hafker, 1993). Sediment samples were mixed with nutrients, then were constantly sparged with air from beneath the soil. There was also a space to allow egress of leachate, although the soil moisture content in these experiments was too low for any leachate to escape. These microcosms will be referred to as 'air-sparged microcosms'.

Four incubation chambers were available, therefore for both the contaminated sediment and the topsoil inocula, the same nutrient amendments were monitored: no added N or P, N supplementation, P supplementation, and N and P supplementation. Each sample was initially supplemented with 'low levels' of N or P, meaning that the quantity of each nutrient was based on the C contributed to the microcosm by sulfolane, using the C:N:P ratio of 100:5:1. Further nutrient amendments were made if biodegradation did not occur after 32 d. No sterile control microcosms were monitored. No loss of sulfolane occurred in the sterile control shake-flask cultures; conditions in these cultures were more favorable to sulfolane biodegradation, therefore it was unlikely that this compound would be lost from the microcosms. Sulfolane has a boiling point of 280 to 285°C (Merck Index, 1968), therefore loss of the compound due to volatilization was unlikely. The setup for an air-sparged microcosm is detailed in Figure 2.3.

The parameters monitored during the air-sparged microcosm experiments were: soil moisture content, sulfolane concentration, total cultureable heterotrophs and sulfolane-degrading bacteria. Sulfolane concentration was monitored using the soil extraction system described in Section 3.5.1. Bacterial populations were monitored using the plate-count method on PAT medium (Section 3.4). Soils consistently lost moisture, particularly the aquifer sediment microcosms, which were incubated in a fume hood due to the smell of some of the contaminant compounds present. To prevent changes in the soil moisture content over time, the microcosms were moistened by the addition of 10 to 50 mL sterile deionized, distilled water, which was thoroughly mixed into the sample. These additions were done approximately weekly (Mueller et al., 1991).

3.5.3.1 Topsoil air-sparged microcosms

The topsoil inocula for air-sparged microcosms were modified with only sufficient N or P to remove the sulfolane that was added to the soil, based on the C:N:P ratio of 100:5:1. The shake-flask cultures inoculated with topsoil were better able to remove sulfolane when modified with this level of N or P than with higher levels.

Sulfolane biodegradation commenced in N-supplemented and N- and P-supplemented microcosms after 9 d of incubation (Figure 3.17B, D). The rate of sulfolane removal from these microcosms was 0.31 and 0.85 mg/L/h, respectively (Table 3.19). These microcosms were refed 200 mg/kg sulfolane at 14 d; by 15 d the N-supplemented and 16 d the N- and P-supplemented microcosms had depleted this sulfolane to below detectable levels (Figure 3.17B, D). Microcosms were reamended with sulfolane to stimulate a further increase in the sulfolane-degrading community. The microcosms were amended with 1 g/kg sulfolane at 16 d; with no appreciable lag time the sulfolane was removed to below detectable levels by 18 d. At 24 and 28 d the microcosms were refed with 1 g/kg sulfolane; both times this amount of sulfolane was removed within 2 d of refeeding the microcosms (Figure 3.17B, D).

Microcosms that were not supplemented with nutrients or were supplemented with P demonstrated lag times of 15 d and 14 d, respectively (Figure 3.17A, C), and degradation rates of 0.35 and 0.56 mg/L/h, respectively (Table 3.19). After the sulfolane had been removed (at 20 d), both microcosms were refed at 22 d with 1 g/L sulfolane. After a lag time of 3 d in both microcosms (31 d after the initial inoculation), sulfolane was completely removed from the P-supplemented microcosm over the following 6 d and the unsupplemented microcosm over a period of 10 d; sulfolane removal was complete in the microcosms after a total of 38 d and 41 d of incubation, respectively (Figure 3.17A, C).

No sulfolane-degrading bacteria (< 10 CFU/g soil) were detectable in any of the topsoil microcosms at the beginning of this experiment (Figure 3.17). This result was not surprising since no sulfolane was detected when unamended topsoil was water extracted. After sulfolane amendment, N-supplemented and N- and P- supplemented microcosms developed sulfolane-degrading communities within 2 d (Figure 3.17). These

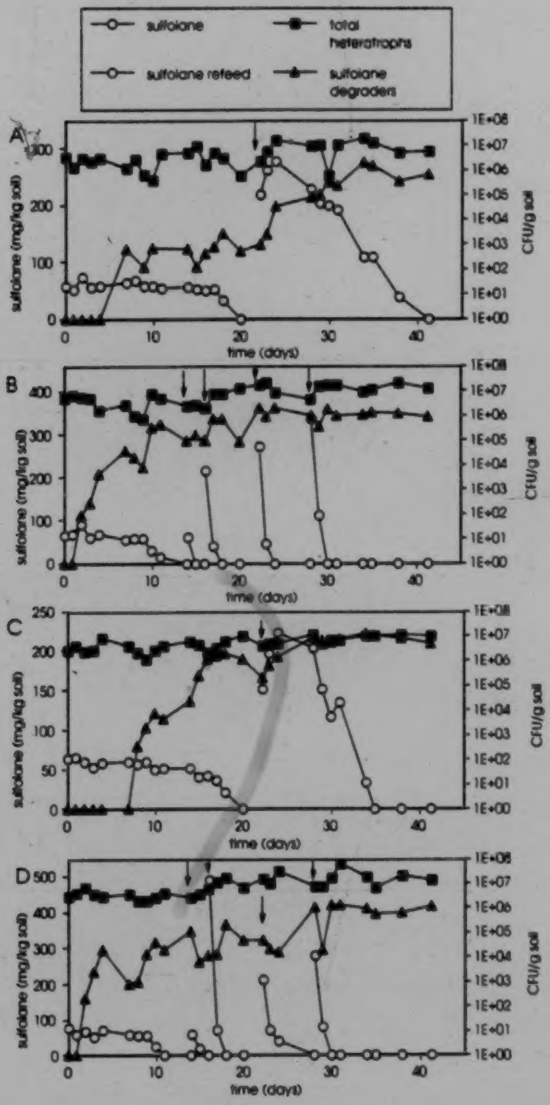


Figure 3.17. Removal of sulfolane from air-sparged microcosms inoculated with topsoil and incubated at room temperature, and the concurrent changes in total heterotrophic and sulfolane-degrading microbial populations. N and/or P was added to account for the sulfolane-C in a sample, according to the C:N:P ratio of 100:5:1. Arrows indicate when microcosms were refed with N, P and sulfolane. Either 200 mg/kg sulfolane or 1 g/kg sulfolane were added. A. Unamended microcosm. B. N-supplemented microcosm. C. P-supplemented microcosm. D. N- and P-supplemented microcosm.

Table 3.19. Removal of sulfolane from air-sparged microcosms inoculated with topsoil, and the total increase in the heterotrophic microbial population and the sulfolane-degrading bacterial population. Sufficient N or P to account for the sulfolane-C in each sample, according to the C:N:P ratio of 100:5:1, was added at the same time as the sulfolane. Microcosms were incubated at room temperature. This table only summarizes the results for the initial round of sulfolane degradation.

nutrients added	initial lag time (d)	initial degradation rate (mg/L/h)	initial change in total heterotrophs (CFU/mg soil)	initial change in sulfolane degraders (CFU/mg soil)
no N or P	15	0.56	ND[a]	10^3
N	9	0.31	ND[a]	10^5
P	14	0.35	10^1	10^6
N and P	9	0.85	ND[a]	10^5

[a] ND: no change in cell numbers was detected.

populations were detectable 7 d before sulfolane biodegradation was observed. Removal of the initial dose of sulfolane resulted in the development of 10^5 CFU/g soil (Table 3.19). Over the period of sulfolane removal, and during the subsequent biodegradation of further increments of added sulfolane, the sulfolane-degrading population in these microcosms increased. The population was first detected at 10^2 to 10^3 CFU/g soil (dry weight); this population increased steadily to approximately 10^6 to 10^7 CFU/g soil, at which point their numbers remained stable for the remainder of the incubation, although three sulfolane amendments of 1 g/kg soil were added after this stable population was reached (Figure 3.17). It is likely that the maximum number of sulfolane-degrading bacteria that could be supported by the availabiliity of sulfolane and nutrients in the soil had been reached.

Over the 41-d incubation period, the number of total cultureable heterotrophs in all four of the topsoil microcosms remained stable, at approximately 10^7 to 10^9 CFU/g soil (dry weight). During the course of sulfolane biodegradation in these microcosms, the sulfolane-degrading bacterial population began to represent a significant portion of the total heterotrophic population, particularly after microcosms had been refed at least once. This data helps support the conclusion that the microbial population of the topsoil microcosms had reached a maximum before the experiment began, and that although the proportion of sulfolane-degrading bacteria increased during the experiment, the total heterotrophic population was unable to increase likewise. The physical niches available for bacterial growth, or the rate of diffusion of O_2, water, substrate, or other essential factors may have limited microbial growth to this level in the topsoil. Given the high levels of organic C present in these samples, the high amounts of N and P naturally present in the soil, and the high numbers of bacteria initially present, it is possible that most of the available organic C was in the form of complex compounds such as humic acid components, that may have been more difficult to degrade than sulfolane. Sulfolane would thus have provided a relatively accessible C source for microbial growth and proliferation. The method for N and P detection in soil did not distinguish in which forms the compounds were present.

Biodegradation of sulfolane began after a longer lag time in the unsupplemented and P-supplemented microcosms; after 18 d and 14 d, respectively (Figure 3.17). Due to time constraints, a second addition of 200 mg/kg sulfolane was not made as it was for the microcosms amended with N; instead 1 g/kg sulfolane was added to these microcosms at 22 d. After a lag time of 2 d, removal of this sulfolane commenced in the

P-supplemented microcosms; this removal was completed by 38 d (16 d after the second dose of sulfolane was given). In contrast, a lag time of 8 d was required by the unsupplemented microcosm, and removal of the compound was not complete until 19 d after the second amount of sulfolane had been added. The sulfolane-degrading bacterial population of the P-supplemented microcosm increased significantly, to approximately 10^7 CFU/g soil (dry weight), by the end of the removal of the initial dose of sulfolane (Figure 3.17, Table 3.18). No increase in bacterial numbers, either of sulfolane degraders or of total heterotrophs, was observed during the removal of the second dose of sulfolane in this microcosm (Figure 3.17).

Changes in the sulfolane-degrading population of the unsupplemented microcosm differed from those of the P-supplemented microcosm, in spite of the similar degradation rates that they demonstrated (Figure 3.17, Table 3.19). The removal of the initial dose of sulfolane resulted in the development of a sulfolane-degrading population of approximately 10^3 CFU/g soil (Table 3.19), however the larger population of 10^7 to 10^8 cells/g that was observed in the other three microcosms early during the incubation period did not appear in the unsupplemented microcosm until removal of the second dose of sulfolane was well underway (Figure 3.17).

In air-sparged microcosms inoculated with topsoil, no nutrient amendment was required in order for sulfolane biodegradation to occur (Figure 3.17A). However, amendment with N decreased the lag time before sulfolane biodegradation commenced, and the addition of P also appeared to have some stimulatory effect on sulfolane degradation. The biodegradation rate did not appear to follow the same trend as the lag times before sulfolane degradation commenced, being faster in the unsupplemented microcosm than in the N- or P-supplemented microcosms (Table 3.19). This was unlike the observations for the Sulfinol® #2 soil (Section 3.5.3.2). Also, the effects of amending the topsoil microcosms with both N and P were not additive. The topsoil microcosms supplemented with N and P showed no difference in lag time before sulfolane biodegradation commenced than microcosms supplemented with N alone, although the degradation rate was faster in the N- and P-supplemented microcosm. The topsoil was rich in C, N and P, well aerated, and had a large intrinsic microbial population, whereas the contaminated sediment was low in N and P, had a lower initial microbial population, and was likely to have been anoxic at the time of sampling. These factors may have accounted for the different effects of nutrient supplementation.

Few changes were observed in the total cultureable heterotrophic population during the 41-d incubation period (Figure 3.17). It is possible that there was little potential for any increase in total heterotrophs before amendment with sulfolane and nutrients; factors other than modification with sources of C, N or P and aeration may have limited this population. It is unlikely that such limitations played a role in the aquifer sediment microcosms, which demonstrated an increase in total heterotrophic bacteria as well as in sulfolane degraders upon mixing and aeration (Figure 3.18).

3.5.3.2 Site 2 contaminated sediment air-sparged microcosms

Contaminated sediment taken from near a Sulfinol® plant building at site 2 was used to inoculate four air-sparged microcosm chambers. These microcosms were monitored frequently during the early part of the experiment; after some time they were monitored on a weekly and then a biweekly basis. Initially, samples were modified with only enough N or P to account for the C contributed by sulfolane, based on the C:N:P ratio of Alef and Nannipieri (1995), however after 32 d of incubation, no loss of sulfolane was observed (Figure 3.18). At this time samples were amended with N and P such that the total C present in the soil was accounted for. Shake-flask experiments suggested that sulfolane biodegradation would eventually occur with the lower levels of nutrients, but that biodegradation would likely begin sooner if higher nutrient levels were provided.

Due to the regular addition of sterile water to the microcosms, soil moisture did not vary greatly over the incubation period (data not shown). It is therefore is not likely that the microbial populations of the microcosms were affected by changes in the soil moisture content.

The N- and P-supplemented microcosm demonstrated a lag time of 3 d after the increased nutrient levels had been administered (Figure 3.18D). After 24 d of incubation with the increased nutrient levels (56 d after the experiment began), the N-supplemented microcosms showed sulfolane-degrading activity; 7 d later (31 d after the nutrient increase) the P-supplemented and unsupplemented microcosms demonstrated activity against this compound (Figure 3.18A-C). The addition of P had no effect on the lag time before sulfolane biodegradation since this microcosm demonstrated the same lag time as the unsupplemented microcosm, although once biodegradation commenced the rate of sulfolane removal was greater in the P-supplemented than in the unsupplemented microcosm. In addition, biodegradation commenced more quickly, and at a greater rate once it began, in the N- and P- supplemented microcosm than in the N-supplemented microcosm (Table 3.20). These results suggest that N was likely the major limiting nutrient for sulfolane biodegradation in this contaminated subsurface sediment, but that P was also limiting, once the greater need for N had been addressed. Similarly, Braddock et al. (1997) observed that the addition of both N and P were required to obtain the highest observed biodegradation rate for hydrocarbons in Arctic soil. When these nutrients were added individually, N appeared to have a strong effect while P appeared to have little or no effect on biodegradation (Braddock et al., 1997).

The water-extractable total organic C (Section 2.8.3.2) in all four microcosms began to decrease before any decrease in sulfolane concentration was observed (Figure 3.18). Water-extractable total organic C was removed fastest from the N- and P-supplemented microcosm, then from the N-supplemented microcosm. In all three microcosms that received some form of nutrient supplementation, water-extractable total organic C was removed to below detectable levels (10 mg/g soil), however in the unsupplemented microcosm water-extractable total organic C removal ceased before sulfolane removal was complete (Figure 3.18). Overall, the removal of water-extractable total organic C paralleled sulfolane removal from the microcosms; being most efficient in those which were able to degrade sulfolane most quickly. The addition of N and P stimulated utilization of water-extractable total organic C, demonstrating that there was a demand for nutrients associated with microbial removal of organic compounds other than sulfolane, thus competition must have occurred between sulfolane-degrading organisms and other heterotrophic bacteria in these microcosms.

The heterotrophic communities demonstrated similar increases in all four microcosms; each community began in the 10^5 to 10^6 CFU/g soil range, and increased to the 10^8 to 10^9 CFU/g range by approximately 25 d of incubation (Table 3.20). No further increases in viable cell counts were observed over the incubation time, although all the bacterial communities were monitored for 165 d (Figure 3.18). In all the supplemented microcosms, the number of total cultureable heterotrophs increased concomitantly with the decrease in total organic C; in the case of the unsupplemented microcosm the total heterotrophs increased before the water-extractable C had decreased noticeably. This could be due to the utilization of C that was not water soluble in this microcosm, or to growth and an increase in cell numbers on a relatively small amount of C in the absence of supplemental N and P.

A sulfolane-degrading bacterial population consisting of approximately 10^3-10^4 CFU/g soil was present in the aquifer sediment at time zero (Table 3.20). This population initially increased over a similar time period to the total heterotrophic population (Figure

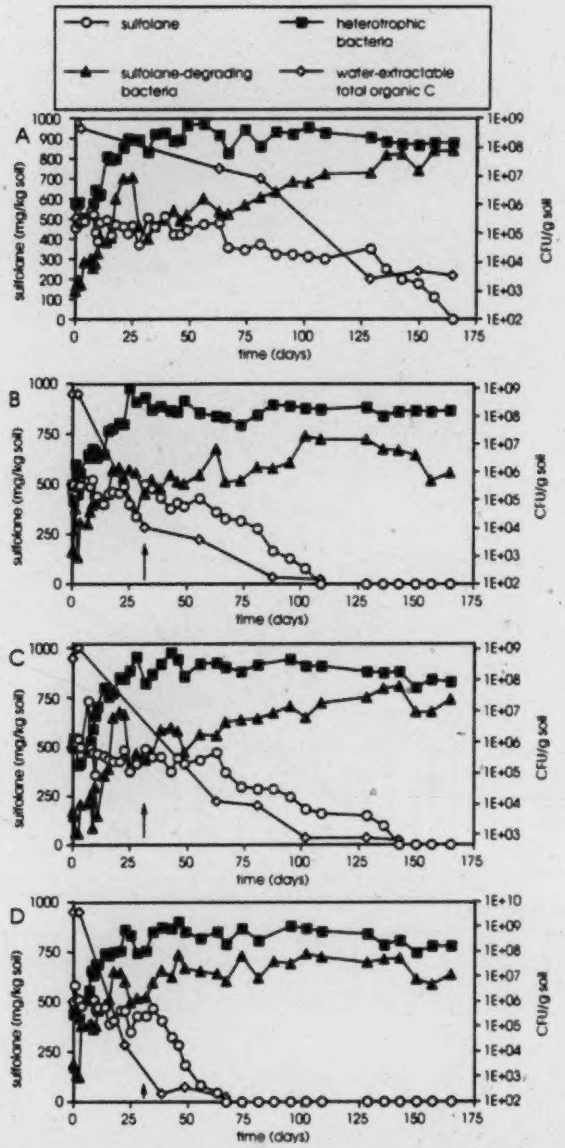


Figure 3.18. Removal of sulfolane from air-sparged microcosms inoculated with sediment from near Sulfinol Building #2 at site 2 and incubated at room temperature, and the concurrent changes in total heterotrophic and sulfolane-degrading microbial populations. N and P were initially added to account for the sulfolane-C, according to a C:N:P ratio of 100:5:1; microcosms were amended with sufficient N or P to account for total C after 32 d of incubation (arrows). A. Unamended microcosm. B. N-supplemented microcosm. C. P-supplemented microcosm. D. N- and P-supplemented microcosm.

Table 3.20. Removal of sulfolane from air-sparged microcosms inoculated with contaminated aquifer sediment taken from near the Sulfinol® Building #2 at site 2, and the total increase in the heterotrophic microbial population and the sulfolane-degrading bacterial population. N and/or P were initially added at low concentrations; the appropriate microcosms were amended to contain high levels of N and/or P after 32 d of incubation. Low N or P refers to sufficient N or P to account for the sulfolane-C in a sample, according to the C:N:P ratio of 100:5:1. High N or P refers to sufficient N or P to account for the total C in a sample. Microcosms were incubated at room temperature.

nutrients added	lag time (d)	degradation rate (mg/L/h)	change in total heterotrophs (CFU/mg soil)	change in sulfolane degraders (CFU/mg soil)
no N or P	63	0.37	10^2	10^5
N	56	0.32	10^3	10^4
P	63	0.19	10^3	10^4
N and P	35	0.96	10^4	10^4

3.18), during the removal of the majority of the water-soluble total organic C in the nutrient-supplemented microcosms. Mixing and aeration provided by the simulated biopile conditions in the microcosms may have stimulated the growth of the total bacterial population, and increases in the sulfolane-degrading population may have fortuitously been a part of the total population increase, rather than the result of specific enrichment.

The sulfolane-degrading bacterial communities in each microcosm increased further later in the experiment, when the total heterotrophic population remained relatively stable (Figure 3.18). The beginning of the second increase in the sulfolane-degrading community was detected in each microcosm within a week of the commencement of sulfolane removal from the microcosm (Figure 3.18). The sulfolane-degrading population may have been enriched initially by growth on other C sources, at the same time as the increase in total heterotrophs. Sulfolane-degrading bacteria were then further enriched by their utilization of sulfolane. During sulfolane degradation, the total heterotrophic community had probably utilized much of the readily degradable C other than sulfolane, therefore this community as a whole was no longer increasing. It is probable that fluctuations in the bacterial population took place after the initial enrichment of heterotrophs; bacteria able to utilize more recalcitrant C compounds likely flourished later in the microcosm incubations, while organisms dependent on easily degraded C compounds would have lacked adequate nutrients for growth and replication. This hypothesis cannot be proved with the data from these microcosms, however it was noted that fewer different colony morphologies were observed later in the incubation time than initially. Fewer fungal colonies were also observed after the landfarming chambers had been incubated for a few weeks (data not shown). Another interesting observation was the differences between the type of sulfolane-degrading organisms that were present in the microcosms amended with various nutrients, based on colony morphology.

The N-supplemented and N- and P-supplemented microcosms mainly showed a single type of sulfolane-degrading organism; this bacterium grew in fairly liquid, spreading, flat, undulate colonies that appeared to produce polysaccharide. After 30 d or more of incubation, one of these colonies could spread far enough to cover the entire nutrient agar plate, overgrowing other microbial colonies present. The unsupplemented and P-supplemented microcosms were dominated by a sulfolane-degrading population which formed circular, flat colonies with a diameter of 2-4 mm, although occasionally one of the colonies that dominated the N-supplemented microcosms would appear. Likewise, occasionally one of the smaller colonies was observed in the N-supplemented microcosms. The colonies that were found in the unsupplemented or P-supplemented microcosms demonstrated the ability to degrade sulfolane within 14 d of plating on PAT medium (Section 3.4), whereas the dominant sulfolane-degrading colonies from the N-supplemented microcosms required at least 21 d to demonstrate sulfolane degradation using PAT medium as an indicator.

From the experimental results there was no way to explain the differences in sulfolane-degrading bacterial colonies that were observed, however some hypotheses can be made. First, it is possible that the sulfolane-degrading bacteria dominant in the N-supplemented microcosms were a species that was unable to utilize the N present in the microcosms, such as ammonium ions. Biodegradation of DIPA results in the release of ammonium ions (Gieg et al., 1999), and as the contaminated soil was obtained from a contaminated aquifer at a Sulfinol® plant site, it is likely that DIPA was present in the aquifer sediment as a co-contaminant with sulfolane. The N added to the soil microcosms was in the form of NO_3^-, which might have been more accessible to this bacterial population than the forms of N available in the aquifer. Second, in the absence of added NO_3^-, a sulfolane-degrading population that was able to utilize the N present in the aquifer sediment, or released from the biodegradation of DIPA and other N-containing contaminants, developed. This population must have utilized sulfolane less readily than the population observed in the N-supplemented sediments, since it took longer to develop. It may have initially utilized compounds other than sulfolane, or been outcompeted by other microbial populations that eventually removed all of some non-sulfolane compound and became inactive, before being able to develop. The near absence of this population in the N-supplemented sediment microcosms suggests that the polysaccharide-producing bacterium was able to successfully compete against the other for substrates such as sulfolane, in the presence of added NO_3^-.

The initial presence of a sulfolane-degrading bacterial population in the subsurface sediment was not surprising; this sediment had been contaminated with sulfolane and other Sulfinol® process chemicals for years before samples were taken for this experiment. The microbial community observed at the beginning of the experiment was almost entirely made up of the smaller bacterial colonies that were later observed to dominate the unsupplemented and P-supplemented microcosms. The existence of this population at the Sulfinol Building #2 site suggested that bioremediation of the site, given the appropriate nutrient and aeration conditions, may be possible.

3.5.4 Comparison of results for shake-flask cultures and air-sparged microcosms

For both sets of shake-flask cultures and air-sparged microcosms, sulfolane biodegradation proceeded under almost all the conditions of nutrient amendment tested. Amendment with N appeared to have the greatest effect in subsurface sediment shake-flask cultures and microcosms (Tables 3.18 and 3.20). Amendment with P had a lesser effect, but still increased microbial activity against sulfolane over the unamended cultures and microcosms. The differences between amendment with different nutrients were somewhat exaggerated in the microcosms, as biodegradation proceeded much more slowly than in the shake-flask cultures. When insufficient N and/or P to account for all the C in the aquifer sediment was added to shake-flask cultures, a longer lag time and slower rate of biodegradation were the result. This being the case, sufficient levels of N and/or P for total C removal were added to microcosms after 32 d of incubation with only enough N and/or P for sulfolane removal; shortly after the high nutrient level amendments biodegradation commenced in these microcosms. The lag period before biodegradation with low levels of nutrients could be estimated at > 32 d but < 62 d in these microcosms; high levels of nutrients were added at 32 d, and biodegradation commenced in the unsupplemented microcosm after 62 d of incubation. Overall, the shake-flask cultures were useful in predicting the behavior of the much slower air-sparged microcosms, with respect to nutrient amendment.

The results from shake-flask cultures inoculated with topsoil material also suggested that N supplementation was more useful in stimulating the development of the sulfolane-degrading bacterial population than P supplementation (Table 3.17). This observation was borne out in the air-sparged microcosms inoculated with topsoil material, where it was noted that sulfolane-degrading populations developed more rapidly in N-supplemented microcosms. The possibility that high levels of N and/or P supplementation somehow decreased the ability of the topsoil population to degrade sulfolane was suggested by the shake-flask culture experiments, however due to time constraints this possibility was not investigated in the air-sparged microcosms.

Previous experiments demonstrated the usefulness of shake-flask experiments in predicting activity against sulfolane in 2.5-L microcosms inoculated with contaminated sediment and groundwater (Section 3.3). Mueller et al. (1991) used landfarming chamber microcosms to predict the potential for biodegradation of pentachlorophenol

(PCP) and creosote compounds in the field. Christensen (1994), Holm et al. (1992), and Nielsen and Christensen (1995a; 1995b) determined that 2.5-L microcosms were useful in predicting *in situ* biodegradation of a number of aromatic compounds in an aquifer sediment. These results, which demonstrated that sulfolane biodegradation in laboratory microcosms could be predicted using shake-flask cultures, may be extrapolated to allow predictions of the potential for microbial activity against sulfolane *in situ*. It is important to realize, however, that the microbial sulfolane degradation observed in the air-sparged microcosms, as in the other laboratory cultures and microcosms, occurred under aerobic, nutrient supplemented conditions. Such conditions are unlikely to occur in a contaminated aquifer without expensive landfarming or forced aeration combined with nutrient amendment in the field.

3.6 Anaerobic microcosms to test the ability of sulfolane to act as a terminal electron acceptor

Tetrahydrothiophene, also known as thiolane, has been detected at a sulfolane spill site in Australia (Kim et al., 1999). Meganathan and Schrementi (1987) discovered that *Escherichia coli* was able to use tetrahydrothiophene-1-oxide as an electron acceptor for the anaerobic oxidation of glycerol or lactate. The product of tetrahydrothiophene-1-oxide reduction was demonstrated to be thiolane, by GC analysis (Schrementi and Meganathan, 1987). The reduction reaction by *E. coli* occurred in a similar manner to reduction of other compounds by this organism, including fumarate, trimethylamine oxide and dimethyl sulfoxide. This reduction pathway required menaquinone (vitamin K2) and a molybdenum-containing enzyme; organisms with mutations in the genes required for either of these factors were unable to utilize tetrahydrothiophene-1-oxide as a terminal electron acceptor (Meganathan and Schrementi, 1987).

Anaerobic sulfolane biodegradation was demonstrated under NO_3^--reducing and Mn(IV)-reducing conditions (Section 3.2.2.2). Sulfolane was also removed from a Fe(III)-reducing microcosm, however no corresponding increase in Fe(II) was observed (Section 3.2.2.2). In all cases, the amount of terminal electron acceptor used was less than half the amount calculated to be required for sulfolane mineralization, suggesting that transformation of sulfolane may have occurred in the anaerobic microcosms. The presence of thiolane at a sulfolane-contaminated site suggested a possible mechanism by which sulfolane transformation may have occurred (Kim et al., 1999). C sources other than sulfolane were present in the contaminated sediments and groundwaters from site

2 (Table 2.2). Likewise, organic compounds other than sulfolane must have been present in the site 1 and site 3 contaminated regions; these were both Sulfinol® plant sites at which sulfolane was mixed with DIPA and sour natural gas during the gas sweetening process. Anaerobic oxidation of some of these compounds, coupled to sulfolane reduction, may have been responsible for some of the sulfolane removal observed in microcosms containing sediment from these sites (J. Coates, personal communication). In order to determine whether sulfolane reduction and the corresponding production of thiolane could be demonstrated, sediment-containing microcosms were amended with sulfolane and/or alternate C sources. Microcosms inoculated with pure cultures of the sulfolane-degrading isolate WPW-S28 (Section 3.7.2.2), *E. coli* K12, *Desulfovibrio vulgaris* variant *oxomicus* or a $SO_4^{=}$-reducing mixed culture from Suncor tailings pond sludge were used to test the ability of these organisms to reduce sulfolane. The experimental methods are described in Section 2.9.2.

3.6.1 Anaerobic sediment-containing microcosms

Two series of sediment-containing microcosms were incubated under anaerobic conditions (Section 2.9.2). The first was used to determine whether volatile, S-containing compounds were produced by samples from a sulfolane-contaminated sediment under anoxic conditions. These microcosms were inoculated with sediment taken from an aquifer near Sulfinol® Building #1 at site 2, which was demonstrated to contain sulfolane-degrading bacteria at the time of sampling (data not shown). The sediment samples were not prepared under anaerobic conditions (Section 2.1.4), however anaerobic technique was used to prepare the microcosms, therefore any O_2 that was present in the sediment would have been depleted by aerobic microorganisms after the microcosms had been sealed.

No new peaks were observed during GC analysis of the headspace gas over a period of 6 mo, suggesting that if sulfolane biodegradation occurred in these microcosms, detectable volatile products, including CO_2 and CH_4, were not produced. If changes in the headspace gas of any microcosms had been observed, sediment from the microcosms would have been sampled in the anaerobic chamber. Extraction of soil slurries with dichloromethane should yield thiolane, if it had been produced (Meganathan and Schrementi, 1987). The amount of water added to the microcosms was insufficient for sampling to analyze sulfolane concentrations in the sediment materials.

The second series of microcosms was designed to determine whether thiolane was produced during the removal of sulfolane from aquifer sediment samples. Site 1 samples were supplemented with sulfolane (250 μg/g soil); little sulfolane was present in the sediment at this site. Half the viable samples were also supplemented with acetate as a C source. Viable site 2 samples were either unsupplemented or were supplemented with acetate. No addition of sulfolane to these samples was required. Sterile control microcosms were made to correspond to each combination of nutrients present. The purpose of this experiment was to determine whether sulfolane biodegradation or biotransformation occurred under anaerobic conditions, and whether acetate could act as an electron donor and sulfolane as a terminal electron acceptor for microbial populations present in this contaminated aquifer.

No loss of sulfolane was observed in these microcosms over a 131-d incubation period. Experiments with the Sulfinol® Building #2 soil in landfarming chamber microcosms and shake-flask cultures demonstrated that biodegradation of sulfolane was stimulated by the addition of N and P sources (Section 3.5), therefore the presence of these nutrients might be required for activity in these microcosms. This possibility was not investigated.

3.6.2 Pure or mixed culture anaerobic microcosms

Pure or mixed culture microcosms were incubated in five different media in order to determine whether these populations were able to utilize sulfolane under anaerobic conditions, and to determine the role that sulfolane played in any anaerobic microcosms in which it was used. Duplicate viable microcosms and one sterile control were incubated for each condition. Two types of media were used to test sulfolane as a C source: these were provided with either NO_3^-- or $SO_4^=$-as terminal electron acceptors. Three types of media provided a mixed C source (acetate, benzoate, glucose, lactate, propionate and yeast extract): these media provided NO_3^-, $SO_4^=$, or sulfolane as a terminal electron acceptor. The mixed culture had previously demonstrated $SO_4^=$-reducing activity and had been enriched from an oil sands tailings pond using acetate as a C source. The pure cultures were WPW-S28 (Section 3.7.2.2), *E. coli* K12, and *Desulfovibrio vulgaris* var. *oxomicus*. Microcosms were monitored visually for reduction of the media (detected by changes in resazurin color) and growth. Sulfolane-containing microcosms were monitored by GC for changes in sulfolane concentration.

After 42 d of incubation, microcosms inoculated with *E. coli* and WPW-S28 had reduced the mixed C source media with NO_3^- or sulfolane as a terminal electron acceptor, and *E. coli* had also reduced the mixed C source medium with $SO_4^=$ as a terminal electron acceptor. No loss of sulfolane was observed in the *E. coli* or WPW-S28 microcosms. No increase in the turbidity of the cultures was observed. It was probable that the microorganisms were able to utilize any small amount of O_2 that was present in the microcosms, thereby reducing the resazurin in the culture supernatants, but they may have been unable to utilize any of the terminal electron acceptors provided. No activity was observed in the $SO_4^=$-reducing mixed or pure cultures, however the inocula for these microcosms contributed only 2% v/v to the total volume of the microcosms, therefore growth was predicted to be slow, and a long lag time was expected. There was no efficient means for monitoring the mixed C source, therefore it was not known whether the inoculum utilized any O_2 remaining in the medium then became dormant, or whether fermentation or anaerobic biodegradation using other compounds as terminal electron acceptors had occurred. Further experimentation is required to determine whether $SO_4^=$-reducing bacteria or microorganisms from sulfolane-contaminated aquifers can reduce sulfolane to thiolane while oxidizing organic C substrates.

3.7 Aerobic cultures

3.7.1 Isolation and description of mixed and pure sulfolane-degrading cultures

Mixed sulfolane-degrading bacterial cultures were derived from contaminated sediment or soil samples by serial enrichment in liquid medium. Highly buffered minimal medium (B+N8P) with sulfolane as a sole C and energy source was used for the enrichments (Section 2.11.1). Mixed cultures were derived from 'cut' and 'till' samples described by Fedorak and Coy (1996), and sediment samples from sites 1, 2 and 3 (described in Table 2.1). The cultures derived from sites 1, 2 and 3 were taken directly from the 28°C shake-flask cultures shown in Figure 3.2. When plated on LB agar and PAT medium, mixed sulfolane-degrading bacterial enrichment cultures generally displayed 10 to 20 different colony morphologies. No fungi or actinomycetes were observed on any of the plates. Usually only one or two colony types in each mixed culture displayed the ability to degrade sulfolane when tested on PAT medium.

Pure sulfolane-degrading bacterial cultures were obtained from mixed cultures. Mixed cultures were streaked on PAT medium; colonies positive for sulfolane-degrading activity were transferred at least 10 times in minimal medium with sulfolane as a sole C and energy source, in order to demonstrate that the ability to degrade sulfolane was real. The purity of these cultures was checked with each serial transfer by streaking on LB agar and on PAT medium. Occasionally, organisms that were unable to degrade sulfolane were found in these theoretically pure cultures, but were not detectable on PAT medium; therefore LB agar was used to separate the sulfolane degraders from these other organisms.

The designations of the mixed and pure cultures discussed in this thesis are given in Table 2.6. A total of 6 mixed and 12 pure cultures were maintained and used for experiments. The cultures which were more active against sulfolane were used more frequently for growth and metabolism experiments. The most-utilized mixed culture was HM8-S28. The first pure culture to be used frequently was HPRK2-S28; until PAT medium was developed this was the only sulfolane-degrading pure culture isolated. However, this culture was not very active compared with other pure cultures. WP1-S28 and HP1-S28 were very active for approximately 1 y after being isolated and were used frequently in experiments during that year. The most active pure culture was WPW-S28; this organism was used for C and S balance and growth experiments and was also selected for identification by 16S rRNA sequence and biochemical means. WPW-S28 was isolated from west plume aquifer sediment taken from site 2 (Table 2.2, Figure 2.1), however this bacterium (or an organism with a similar colony morphology) was also the dominant sulfolane-degrading bacterial species in the east contaminated plume aquifer sediment at this site. A second, less dominant species in the east plume aquifer sediment did not have sulfolane-degrading abilities in liquid medium, although it gave a positive result on PAT medium. No other colonies able to degrade sulfolane were isolated from the west plume aquifer sediment.

3.7.2 Growth of aerobic mixed and pure cultures on sulfolane

3.7.2.1 Aerobic mixed sulfolane-degrading cultures

Mixed sulfolane-degrading cultures were grown on sulfolane in a variety of media. In B+NP or $SO_4^=$-free media, the removal of approximately 50 mg/L sulfolane from a culture resulted in a pH drop from approximately pH 7.0 to between pH 3.5 and 4.0, which inhibited any further growth (measured by OD_{600}) or sulfolane degradation in these cultures (data not shown). Buffering with 5 g $CaCO_3$ in 200-mL cultures or by using B+N8P medium allowed the removal of up to 1 g/L sulfolane; approximately 500 mg/L with no detrimental drop in the pH and the remainder of the sulfolane after the culture pH had begun to decrease. A typical example of the growth of a mixed culture with sulfolane as a sole source of C and energy is shown in Figure 3.19. Similar results were obtained when sulfolane was also used as the sole source of S for the cultures.

Lag times for sulfolane biodegradation by mixed cultures varied. The factors that demonstrated the most strongly marked effects on lag times before biodegradation commenced were the age and size of the inoculum in the new culture. The shortest lag times were observed when a 10% inoculum (v/v in the new culture) was transferred within a week of sulfolane removal from the previous culture medium. Inoculum sizes representing less than 5% of the total culture volume always resulted in lag times of > 2 wk. Lag times of 2 wk to 1 mon were also not uncommon if cultures had been left 1 mon or more before transfer. Once biodegradation of sulfolane commenced, the rate of degradation was similar, regardless of the lag time (data not shown). This suggested that the sulfolane-degrading organisms remained dormant, but once stimulated they were able to grow and divide rapidly.

3.7.2.2 Aerobic pure sulfolane-degrading cultures

Pure sulfolane-degrading cultures demonstrated similar or longer lag times than those of the mixed cultures, particularly with respect to the age of the culture and the inoculum size. The inoculum size was more important with pure cultures than with mixed cultures; an inoculum size of at least 10% of the total culture volume was required to prevent long lag times. Unlike the mixed cultures, each pure culture demonstrated a different level of activity against sulfolane, as measured by the amount of sulfolane removed from a culture over a given time and the corresponding increase in OD_{600} (Table 3.21). The growth, sulfolane utilization and changes in pH for each culture are shown in Figures B2 and B3 (Appendix B). The most active sulfolane-utilizing pure culture was WPW-S28 (Figure 3.20).

3.7.3 Effect of sulfolane analogs on the growth of aerobic pure and mixed cultures

The ability of pure and mixed sulfolane-degrading cultures to grow on sulfolane analogs, or to modify or degrade sulfolane analogs while growing on sulfolane, was tested. Should certain modifications of sulfolane block biological activity against the compound, the first step of biodegradation could be hypothesized. Five sulfolane analogs: 3-methylsulfolane, 2,4-dimethylsulfolane, 3-hydroxysulfolane, sulfolene, and butane sultone were tested. The structures of these compounds are shown in Figure 2.4. Controls to which both sulfolane and a sulfolane analog were added demonstrated whether any sulfolane analogs were toxic to the bacterial cultures, or were able to irreversibly block the sulfolane degradation pathway.

Mixed and pure cultures were able to utilize 3-methylsulfolane and 2,4-dimethylsulfolane as a sole source of C and energy (Table 3.22). The mixed culture was also able to degrade 3-hydroxysulfolane, however neither pure culture that was tested could utilize this compound. Neither the pure nor the mixed cultures were able to

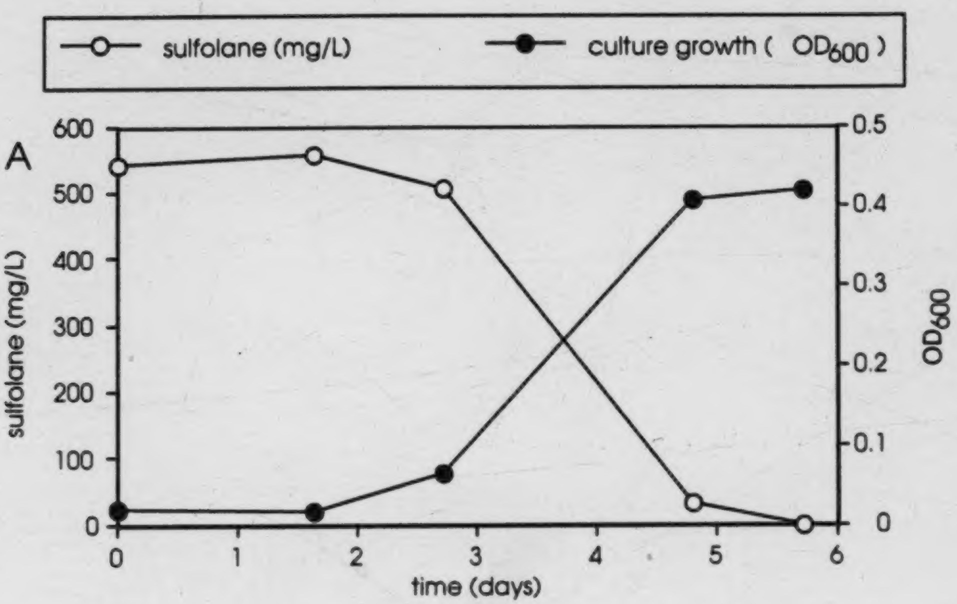


Figure 3.19. Growth of the mixed culture HMRW3-S28 on sulfolane in a highly buffered medium. This is a typical example of growth of a mixed culture with sulfolane as the sole C and energy source. Similar results were obtained when sulfolane was also utilized as a source of S.

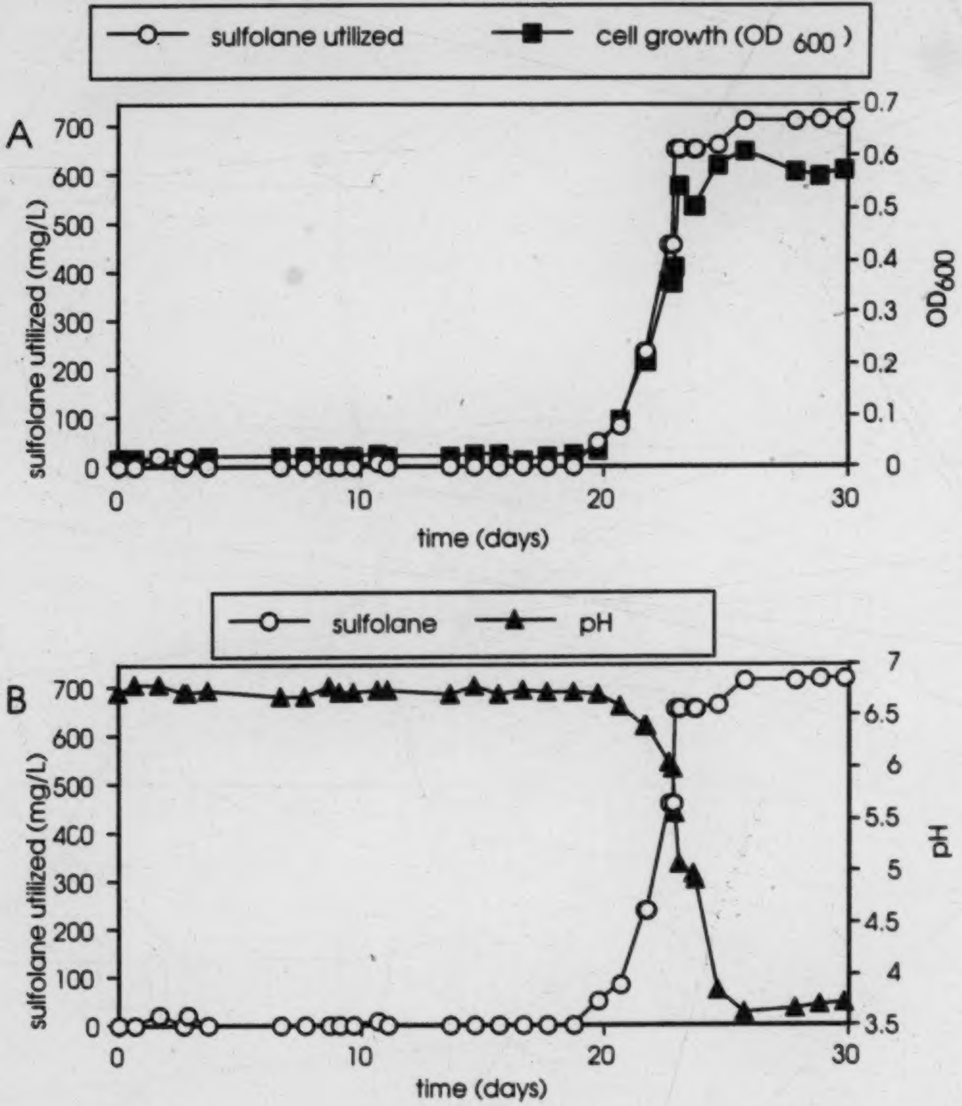


Figure 3.20. Growth of the pure culture WPW-S28 on sulfolane in B+N8P medium in a 200-mL shake-flask culture. A. Total sulfolane utilized and the corresponding increase in OD$_{600}$ in the culture. B. Total sulfolane utilized and the corresponding decrease in pH. Note that although B+N8P medium is highly buffered, the pH decreased rapidly after the removal of approximately 700 mg/L sulfolane, and that culture growth ceased after the decrease in pH occurred.

Table 3.21. Utilization of sulfolane over time by pure bacterial cultures grown in B+N8P medium. Culture growth was measured using OD_{600}. Total sulfolane consumed was calculated based on individual feedings of 200 mg/L sulfolane. The time for degradation was calculated by subtracting all lag times from the total time over which degradation occurred. The pH was measured at the beginning and the end of the experiment. Cultures were incubated at 28°C, except HP5-S8, which was incubated at 8°C.

culture designation	initial lag time (h)	degradation time (h)	total sulfolane used (mg/L)	total increase in OD_{600}	total decrease in pH
HP1-S28	64	456	287	0.078	0.58
HP5-S8	16	384	578	0.493	3.11
HP8-S28	88	531	134	0.046	0.30
HP81-S28	16	627	481	0.073	0.94
WP1-S28	40	264	278	0.118	1.06
WPES-S28	16	699	937	0.305	3.79
WPU-S28	64	432	645	0.318	2.16
WPW-S28	376	118	719	0.589	3.17
WPWS-S28	16	699	414	0.072	0.64

Table 3.22. Biodegradation of sulfolane analogs by one mixed and two pure sulfolane-degrading cultures. Biodegradation was considered to occur if a decrease in the sulfolane-analog concentration was observed concomitant with an increase in the OD_{600} of the culture. Cultures were incubated at 28°C for 1 mon. In a parallel study, sulfolane was degraded in the presence of all the sulfolane analogs tested.

sulfolane analog	biodegradation of sulfolane analog observed		
	HM8-S28 mixed culture	HPRK2-S28 pure culture	HP1-S28 pure culture
3-methylsulfolane	yes	yes	yes
2,4-dimethylsulfolane	yes	yes	yes
3-hydroxysulfolane	yes	no	no
sulfolene	no	no	no
butane sultone	no	no	no

degrade sulfolene or butane sultone (Table 3.22). The mixed culture used for these tests was HM8-S28. Two pure cultures were used to test the sulfolane analogs: HP1-S28 and HPRK2-S28. Both pure cultures showed similar degradative abilities, as well as similar growth characteristics on the compounds tested. In the presence of sulfolane, the sulfolane analogs were utilized more slowly than sulfolane itself, however both compounds were eventually removed. An example of this is shown for 3-methylsulfolane degradation by HM8-S28 in Figure 3.21. Growth of the pure cultures on sulfolane analogs was much slower than the growth of the mixed culture on the same compound, and the pure cultures required a longer lag time before growth commenced.

The abilities of one mixed and two pure bacterial cultures to degrade sulfolane analogs are summarized in Table 3.22. A hydroxyl group on one compound and a methyl group on another blocked the 3-position of sulfolane; the former was degraded by a mixed culture but untouched by two pure sulfolane-degrading cultures, while the latter was degraded by both mixed and pure cultures. Sulfolene, which is unsaturated between C 3 and 4 in sulfolane, was not degraded, yet 2,4-dimethylsulfolane was easily used by all three cultures tested, and this was a much bulkier molecule. Butane sultone, which was predicted to be a potential intermediate in aerobic sulfolane degradation, was also not degraded. It was impossible to form any useful conclusions about the pathway for aerobic sulfolane degradation from this information. The structure of the sulfolane analogs tested did not appear, at least superficially, to be important for degradability of the compounds. It was not determined whether cells could take up sulfolene or butane sultone; if the compounds were not easily taken into cells then their catabolism may not have been possible. Further experiments are required to determine the degradation pathway for sulfolane; once this has been elucidated it might be possible to explain the degradation or lack of degradation of the sulfolane analogs tested here, and to predict the potential for degradation of other similar compounds.

3.7.4 Growth of E. coli K12 thiophene-degrading mutants on sulfolane

Eight *E. coli* K12 mutants capable of degrading thiophene (Juhl and Clark, 1990) obtained from D. Clark are described in Table 2.7. Four of these strains (NAR11, MW90, MW96 and MW100) had sulfone oxidase activity against sulfolane (Juhl and Clark, 1990); as well, these strains appeared positive for sulfolane utilization on tetrazolium indicator plates (Juhl and Clark, 1990). The mutant and wild type organisms were grown with sulfolane in liquid culture, in the presence or absence of alternate C sources (glucose or acetate). All the strains grew in the presence of the alternate C sources,

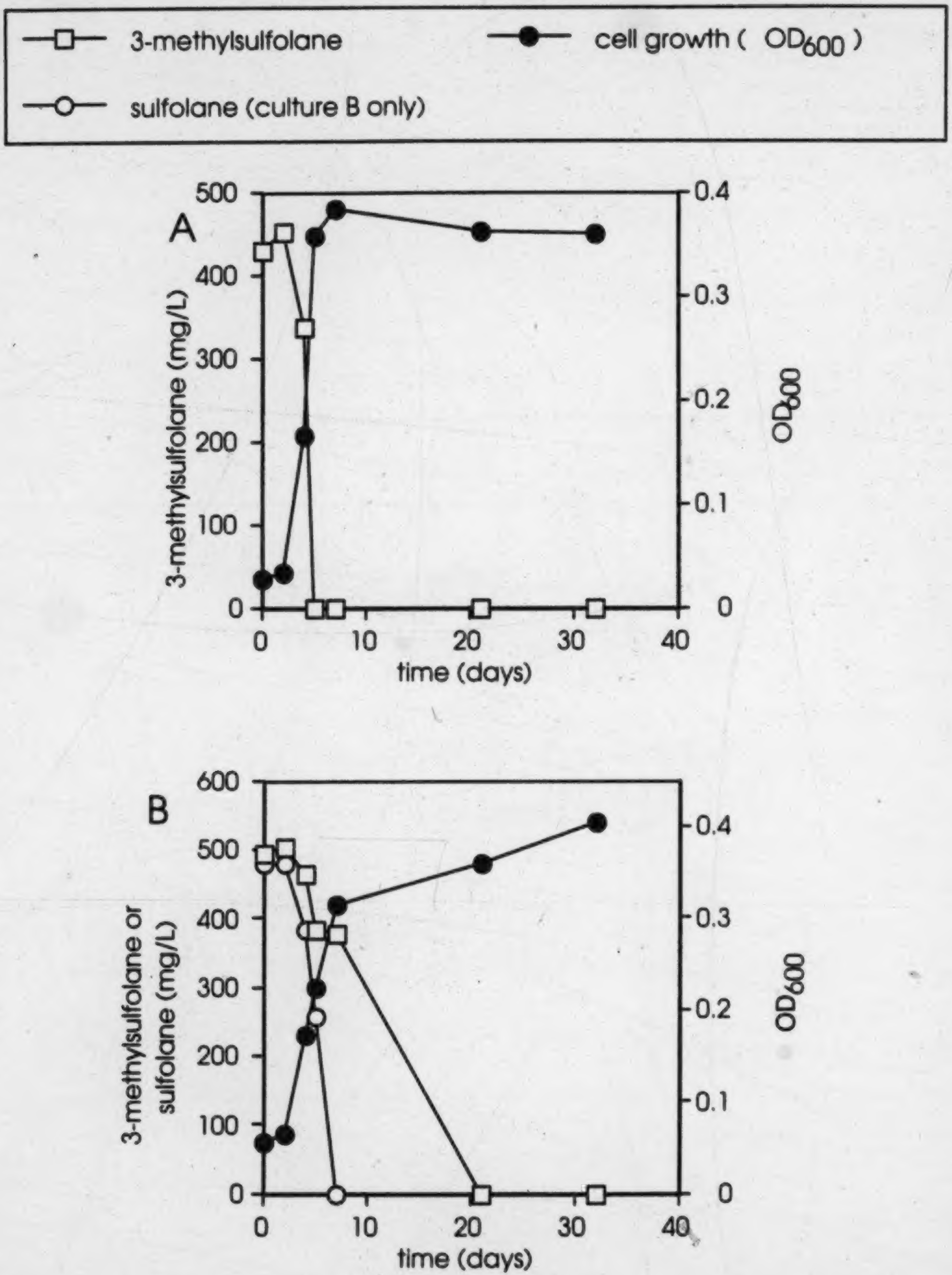


Figure 3.21. Growth of the mixed culture HM8-S28 on A. 3-methylsulfolane or B. 3-methylsulfolane and sulfolane as a sole source of C.

however no sulfolane removal was detected in any of the cultures, even after > 1 mon of incubation. Juhl and Clark (1990) did not test the ability of the *E. coli* strains to grow on sulfolane; it is possible that although the compound stimulated sulfone oxidase activity in some of the thiophene-degrading mutant strains, the enzyme did not affect sulfolane. Tetrazolium is a redox indicator dye and may have been affected by the utilization of other components in the medium, or by a product of sulfolane modification by the *E. coli* strains, therefore may have given a false positive result for sulfolane degradation on tetrazolium indicator agar. The authors did not extensively investigate the degradation of sulfolane or other S-containing compounds that were screened with the *E. coli* mutants; their main purpose was to characterize the means by which these mutants degraded thiophene (Juhl and Clark, 1990).

3.8 Carbon and sulfur balance experiments

C and S balance experiments were carried out to determine the extent of mineralization of sulfolane to CO_2 and $SO_4^{=}$ and its conversion to biomass by mixed and pure cultures enriched from contaminated aquifer sediments, under aerobic conditions. This mineralization is summarized in the first equation in Table 3.5. Anaerobic biodegradation of sulfolane was poor, in the cases where it was observed, therefore aerobic mineralization experiments appeared to be more useful for predicting sulfolane biodegradation products. Complete mineralization of sulfolane would suggest that if an aquifer could be infused with O_2 and the appropriate nutrients, bioremediation of the site might be achievable. Should mineralization of sulfolane be incomplete, it would be necessary to isolate and identify the remaining products of sulfolane catabolism in order to predict their behavior and toxicity in contaminated aquifers.

3.8.1 Method development: serum bottle, shake-flask and fermentor cultures

Three methods were tested in order to determine the best method for sulfolane mineralization experiments. Cultures were grown in shake-flasks, serum bottles, or in a 14-L fermentor to determine which incubation method would yield the most reliable results. Radiolabeled sulfolane was not available; therefore alternate methods to detect CO_2 and $SO_4^{=}$ were required.

3.8.1.1 Oxygen requirements for sealed cultures

Shake-flask and serum bottle cultures were sealed to prevent release of CO_2 generated from sulfolane degradation. The amount of O_2 required for mineralization of a given amount of sulfolane therefore had to be present in the headspace of each culture;

this was 6.5 mol O_2 for the mineralization of 1 mol sulfolane. The maximum amount of sulfolane added to sealed cultures was calculated based on this information.

3.8.1.2 Development of a serum bottle method to determine a carbon and sulfur balance for sulfolane degradation

The mixed culture HMRW3-S28 and the pure culture HPRK2-S28 were incubated with sulfolane or acetate in the initial serum bottle experiments (Section 2.12.1.1). The pure culture did not grow under these experimental conditions. The mixed culture grew on both sulfolane and acetate; approximately 80% of the acetate-C was converted to CO_2, as measured by GC. Little CO_2 was detected in the headspace of the sulfolane-degrading cultures. Due to the limitations in culture size for serum bottle experiments, replicate cultures were required for CO_2 and $SO_4^=$ analysis, therefore methods which yielded larger culture volumes were explored.

3.8.1.3 Fermentor culture to determine a carbon and sulfur balance for sulfolane degradation

In order to obtain samples from which both CO_2 and $SO_4^=$ could be measured simultaneously, a large culture volume was required. A 14-L fermentor was used to incubate a 10-L culture of the pure sulfolane-degrading culture HPRK2-S28 (Section 2.12.1.2). This large culture size allowed continuous sampling to monitor OD_{600} and sulfolane concentration, as well as providing sufficient material for $SO_4^=$ analysis and to determine the total C and S yield in the cells. The increase in biomass was measured by the ratio of cell dry weight in a constant volume of medium at the beginning and the end of the incubation. The CO_2 produced by the culture was trapped and quantified. The advantages of the fermentor system were the large culture supernatant and the automatic controls available; temperature, pH, air intake, cell growth and sulfolane concentration could all be monitored regularly throughout the experiment. The major disadvantages were the large inoculum and amount of medium required for a single experiment and the inability to do replicate experiments simultaneously.

The pure culture HPRK2-S28 converted 10% of the sulfolane-C in the medium to CO_2 and 65% of the S in sulfolane to $SO_4^=$. Approximately 20% of the C from sulfolane was converted to biomass. Two replicate experiments were done, and the results were similar for both. Dried cells from the first fermentor run were found to be 9.7999 % N, 36.175 % C, 5.916 % H and 0.000 % S (Analytical Service Laboratory, Dept. of Chemistry, University of Alberta, Edmonton, AB). The dried cells were retained and used as a C standard for biomass C measurements using a total organic C analyzer. The fermentor method for determining sulfolane mineralization was only used twice; this method was acceptable for testing a single set of culture conditions but was too cumbersome for experiments in which more parameters were tested simultaneously.

3.8.1.4 Shake-flask method to determine a sulfur balance for sulfolane degradation

Different shake-flask experiments were done using mixed and pure cultures of sulfolane-degrading bacteria to determine the extent of sulfolane mineralization during growth (Section 2.12.1.3). The amount of CO_2 and soluble organic C produced was not determined due to problems with the techniques for quantifying CO_2 in these cultures, however the production of $SO_4^=$ from sulfolane was measured. Typical examples were 79%, 74%, and 57% of sulfolane-S released as $SO_4^=$ in the cultures HM8-S28, HM3-S8 and WP1-S28, respectively.

3.8.1.5 Summary of methods for determining a carbon and sulfur balance for sulfolane degradation

Three methods were evaluated for their usefulness in determining the extent of sulfolane mineralization by sulfolane-degrading bacterial cultures. Although it was originally felt that replicate cultures would be less accurate than a single, large culture, the complexity of operating a large system demonstrated more potential for error than the employment of replicate cultures. Additionally, CO_2 analysis was only reproducible when done by GC; in order to do this the culture supernatant had to be acidified to force CO_2 into the headspace. It was felt that analysis of CO_2 directly in the incubation vessel would leave less chance for errors. The acidification of a culture was likely to lyse the cells, thereby complicating the results of $SO_4^=$ and soluble organic C analyses. The use of replicate cultures avoided this problem. Serum bottle cultures were easiest to use, and gave the most reproducible results. These cultures were small, easily sealed, and easily sampled; > 400 cultures could be incubated simultaneously. Shake-flask cultures were most useful for experiments which monitored only $SO_4^=$ production; these cultures could be used for time course experiments and did not need to be sealed.

3.8.2 Carbon and sulfur balance for mixed and pure sulfolane-degrading cultures

Carbon and S balance experiments were done to determine the extent of sulfolane degradation by various pure and mixed sulfolane-degrading cultures. All these experiments were done using the serum bottle method of incubation (Section 2.12.1.4). Cultures were generally 50 mL, in 158-mL serum bottles, and were given between 50 and 300 mg/L sulfolane. Incubations were carried out in $SO_4^=$-free medium; the pH of cultures was adjusted using sterile 0.2 M NaOH. Rather than changing the volume of cultures by removing samples to monitor sulfolane concentration and cell growth, replicate test cultures were sampled, and were also used to determine the correct amount of NaOH to add for neutralization of the cultures during growth. Test cultures therefore contained bromthymol blue pH indicator; samples for analysis did not contain this compound as it affected the level of soluble organic C in samples and might be degraded by some sulfolane-degrading organisms. Activity against bromthymol blue pH indicator by sulfolane-degrading bacteria was previously observed (Section 3.4.4).

Initial serum bottle experiments with 300 mg/L sulfolane demonstrated that the mixed culture HM8-S28 converted sulfolane-C to 47% CO_2, 8% soluble organic C and 29% biomass, with 16% of C not detected, and 102% of sulfolane-derived S was detected as $SO_4^=$. In the same experiment, the pure culture WP1-S28 converted C from sulfolane to 23% CO_2, 53% soluble organic C, 16% biomass, and 8% of C was not detected, while 53% of the S from sulfolane was detected as $SO_4^=$. These results suggested that more sulfolane was mineralized in a mixed than in a pure culture of sulfolane-degrading bacteria. Mineralization of organic compounds is frequently more extensive when done by a consortium of bacteria, rather than a pure culture, however for a compound as simple as sulfolane this result seemed slightly unusual. Once the ring structure of sulfolane has been opened, it would be a straight chain compound that should be easily metabolized by any single organism. No explanation for the incomplete mineralization of sulfolane could be produced from these results.

Sulfolane degradation by four cultures was investigated at four concentrations of sulfolane (Table 3.23). The cultures were the two previously tested: HM8-S28 and WP1-S28, as well as one other mixed (HM5-S8) and one other pure (HP1-S28) culture. For each measurement at each concentration, three replicate cultures were analyzed. The most reproducible results were obtained at sulfolane concentrations of 100 and 200 mg/L; cultures given 50 or 300 mg/L demonstrated more variability between replicate samples. These problems appeared to be chiefly due to variations in CO_2 analysis. At low sulfolane concentrations in particular the level of CO_2 produced may have been too low for accurate detection. Cripps (1973) similarly attributed the incomplete recovery of C from thiophen-2-carboxylate to an incomplete recovery of CO_2 from test cultures.

Conversion of sulfolane-C to CO_2 and sulfolane-S to $SO_4^=$ appeared to be more extensive in the mixed than in the pure cultures (Table 3.23), as noted with the previous experiment. Production of biomass did not appear to be more extensive in the mixed than in the pure cultures, yet the OD_{600} for mixed cultures was generally substantially higher than that of the pure cultures (data not shown). The difference between the mixed and pure cultures mainly lay in the amount of soluble organic C remaining in the medium after sulfolane removal was complete. This was much higher in the pure cultures (24 to 39% in HP1-S28, 28 to 81% in WP1-S28) than in the mixed cultures (2 to 12% in HM8-S28, 8 to 18% in HM3-S8). The recovery of C was never 100%; in seven sets of replicates the recovery of C was < 100% and in nine sets, the recovery of C was > 100% (Table 3.23). This was likely due to errors generated by using a total of four different techniques to measure the amount of C present in each sample: the sulfolane concentration in each sample was determined at the beginning of each experiment to measure added C, and at the end of the experiment the changes in CO_2, soluble organic C and biomass C were measured.

A final C and S balance experiment was done to determine whether CO_2, $SO_4^=$ and soluble organic C compounds were released and biomass had increased immediately as 100 mg/L sulfolane was degraded, or whether there was a lag time before any products of sulfolane utilization by a culture were observed. The pure culture WPW-S28, which grew vigorously on sulfolane, was used for this time course. Triplicate samples were analyzed for each time point.

Sulfolane degradation commenced by 1 d, and the compound was completely lost after 2 d of incubation (Figure 3.22). Sulfate production was not simultaneous with sulfolane degradation; after 1 d 78% of the added sulfolane was removed from the cultures, but only 26% of sulfolane-S was detected as $SO_4^=$. The soluble organic C decreased over the entire incubation period, and CO_2 increased over this time. Biomass increased for the first 72 h of incubation, and decreased by 11 d of incubation, as expected for a bacterial culture that reached stationary phase after 2 d (Figure 3.22).

Overall, these results suggested that release of $SO_4^=$ from sulfolane occurred shortly after removal of the compound from the culture medium, which implicated ring cleavage as an early step in sulfolane degradation. After the opening of the ring and release of $SO_4^=$, the remainder of sulfolane would likely have consisted mainly of C. Part of this C was converted to CO_2 and biomass over time; the remainder appeared to be in some form of soluble organic C. It is unlikely that any of this C was CO_2 trapped in the aqueous medium as HCO_3^-; samples for CO_2 analysis were acidified to pH ≤ 2, which would force HCO_3^- into the headspace as CO_2. Furthermore, samples analyzed for soluble organic C were acidified with concentrated H_2SO_4 and purged with N_2 to remove any CO_2 present, at the time of C measurement.

Table 3.23. Conversion of sulfolane to CO_2, biomass, total organic C and $SO_4^{=}$ by two mixed and two pure cultures. Cultures were incubated for 30 d to ensure complete biodegradation of sulfolane. Conversion is described as the per cent of total C or S added as sulfolane at time zero that was detected in each form.

culture identity	sulfolane added (mg/L)	$SO_4^{=}$ (%)	CO_2 (%)	soluble organic C (%)[a]	biomass C (%)	total C recovery (%)
HP1-S28	50	50 ± 5	4 ± 6	39 ± 2	28 ± 2	71
(pure)	100	71 ± 5	42 ± 0	28 ± 1	17 ± 0	87
	200	78 ± 5	45 ± 1	24 ± 6	17 ± 1	86
	300	65 ± 3	82 ± 0	35 ± 8	33 ± 1	151
WP1-S28	50	40 ± 1	0 ± 0	81 ± 15	23 ± 2	103
(pure)	100	55 ± 4	40 ± 1	53 ± 3	23 ± 2	115
	200	39 ± 1	42 ± 1	47 ± 6	20 ± 3	109
	300	44 ± 4	44 ± 0	28 ± 1	14 ± 8	87
HM8-S28	50	85 ± 4	58 ± 2	12 ± 2	22 ± 3	91
(mixed)	100	81 ± 0	60 ± 1	6 ± 1	20 ± 2	86
	200	74 ± 1	74 ± 1	2 ± 1	19 ± 2	96
	300	65 ± 1	79 ± 1	3 ± 0	18 ± 1	101
HM3-S8	50	69 ± 2	81 ± 3	18 ± 0	8 ± 3	106
(mixed)	100	97 ± 3	80 ± 1	8 ± 1	26 ± 1	113
	200	90 ± 1	99 ± 1	13 ± 4	4 ± 1	117
	300	96 ± 4	84 ± 1	14 ± 5	5 ± 1	103

[a] All detectable sulfolane had been removed from the cultures at the time of analysis, therefore no sulfolane contributed to the reported soluble organic C.

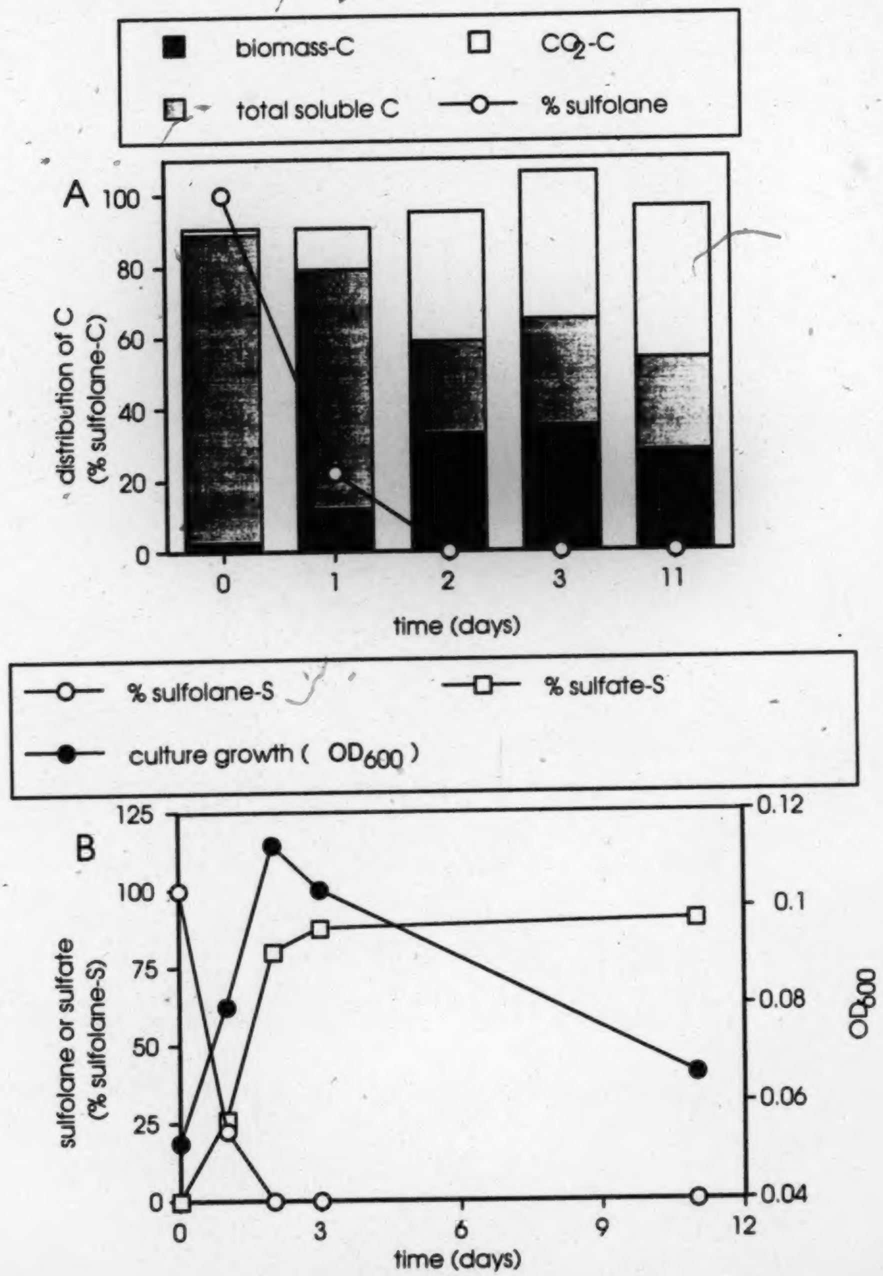


Figure 3.22. Sulfolane degradation by triplicate samples of the pure culture WPW-S28. A. Sulfolane conversion to soluble organic C, biomass C and CO_2. Standard deviations were all ≤ 1.1 % for C analysis. B. Sulfolane conversion to $SO_4^=$ and changes in the OD_{600} of WPW-S28 cultures. Standard deviations were ≤ 2 %.

Several authors have done C or C and S balance experiments on compounds similar in structure to sulfolane: 1,4-dioxane, thiophen-2-carboxylate and propan-2-yl sulfonate, or for more complex compounds, such as isopimaric acid. Parales et al. (1994) determined that a 1,4-dioxane-degrading actinomycete converted 60% of dioxane to CO_2. These authors found no dissolved organic C in the culture supernatant, therefore they assumed that the remainder of the C from dioxane was converted to biomass. This amount of C incorporation into biomass was greater than in the sulfolane-degrading cultures, which converted a maximum of 33% of sulfolane-C to biomass (Table 3.23). However, none of the organisms present in the mixed or pure sulfolane-degrading cultures were actinomycetes. This difference in the composition of the cultures may have accounted for the different conversion to biomass; actinomycetes have different growth characteristics than most other bacterial types.

Cripps (1973) demonstrated that an unidentified organism converted 74% of thiophen-2-carboxylate-C to CO_2, approximately 4% to biomass C and 100% of the S from this compound to $SO_4^=$. The results of Cripps (1973) were similar to those observed for mixed culture sulfolane biodegradation, while the CO_2 production reported by Parales et al. (1994) more closely resembled the pure culture results obtained for sulfolane degradation. Cripps (1973) noted that ^{14}C detection was incomplete, and stated that this was likely due to incomplete CO_2 trapping in NaOH, therefore the extent of thiophen-2-carboxylate mineralization may have been greater than the 74% reported. In devising a method for detecting CO_2 from sulfolane degradation, trapping with NaOH gave poorer yields than GC analysis. This finding was borne out by the observations of Cripps (1973). However, other analytical difficulties may have contributed to the incomplete recovery of ^{14}C from thiophen-2-carboxylate by Cripps (1973). Only 4% of the added ^{14}C was detected in the biomass in these cultures. This relatively small amount of C incorporation might not account for the actual increase of biomass in the cultures, therefore some of the ^{14}C incorporated into the cells may not have been detected.

Experiments to determine the mineralization of a more complex structure, isopimaric acid, demonstrated that two bacterial isolates were both able to convert only one third of this compound to CO_2; the remainder was recovered as one third biomass and one third dissolved organic C (Wilson et al., 1996). Parales et al. (1994) and Wilson et al. (1996) both measured CO_2 by GC-TCD. This method was also used for CO_2 measurement in sulfolane-degrading cultures. Parales et al. (1994) did not report a complete mass balance for 1,4-dioxane. Wilson et al. (1996) were able to recover slightly more than 100% of the C added in the form of isopimaric acid in one culture, and 100% of the added C in the second culture tested. Overall, the sulfolane mineralization experiments reported here appeared to give similar recoveries as those observed by Wilson et al. (1996), and similar CO_2, $SO_4^=$ and biomass yields to those reported in the literature for thiophen-2-carboxylate, isopimaric acid and 1,4-dioxane.

3.8.3 Sulfate release time course for mixed and pure cultures

The major S-containing product from sulfolane degradation by a variety of mixed and pure bacterial cultures, $SO_4^=$, accounted for 39% to 102% of S released from sulfolane (Table 3.23). The pure culture WPW-S28 released $SO_4^=$ nearly simultaneously with sulfolane removal from the culture, while the mineralization of C from sulfolane to CO_2 by this culture proceeded more slowly after the parent compound had disappeared (Figure 3.22). Sulfate release during sulfolane degradation was monitored in mixed and pure cultures (Section 2.12.2), to determine whether $SO_4^=$ production corresponded immediately with the disappearance of sulfolane, or whether the timing of $SO_4^=$ release varied depending on the bacterial culture using the compound.

Duplicate cultures demonstrating the disappearance of sulfolane and the corresponding increase in cell numbers and $SO_4^=$ are shown in Figure 3.23 for two pure and one mixed culture. The mixed culture, HM8-S28, demonstrated complete (99% and 106%) recovery of S from sulfolane as $SO_4^=$. A slight lag was observed between the time of sulfolane removal and the time for the recovery of $SO_4^=$ (Figures 3.23A, B). A similar lag time between sulfolane removal from the culture supernatant and $SO_4^=$ release was observed for HP1-S28 and WP1-S28 (Figures 3.23C-3.23F). The pure culture WPW-S28 also demonstrated $SO_4^=$ release from sulfolane after a short lag, rather than immediately upon sulfolane degradation (Figure 3.22B). The major differences between the mixed and pure cultures were that cell growth was faster and a higher final OD_{600} was observed in the mixed culture, concomitant with faster removal of sulfolane and a more complete release of $SO_4^=$. The amount of $SO_4^=$ released in the pure cultures was 77% to 85% for HP1-S28 and 49% to 56% for WP1-S28.

Crescenzi et al. (1984) demonstrated stoichiometric release of $SO_4^=$ from propan-2-yl sulfate by *Pseudomonas syringae* concomitant with degradation of the compound by this culture. Similarly, Cripps (1973) observed stoicheometric $SO_4^=$ release from thiophen-2-carboxylate concomitant with removal of the compound from the culture supernatant by an unknown organism, with a 90% recovery of total S. Cultures grown by Cripps (1973) were incubated for a total of 3 d; Crescenzi et al. (1984) grew cultures for

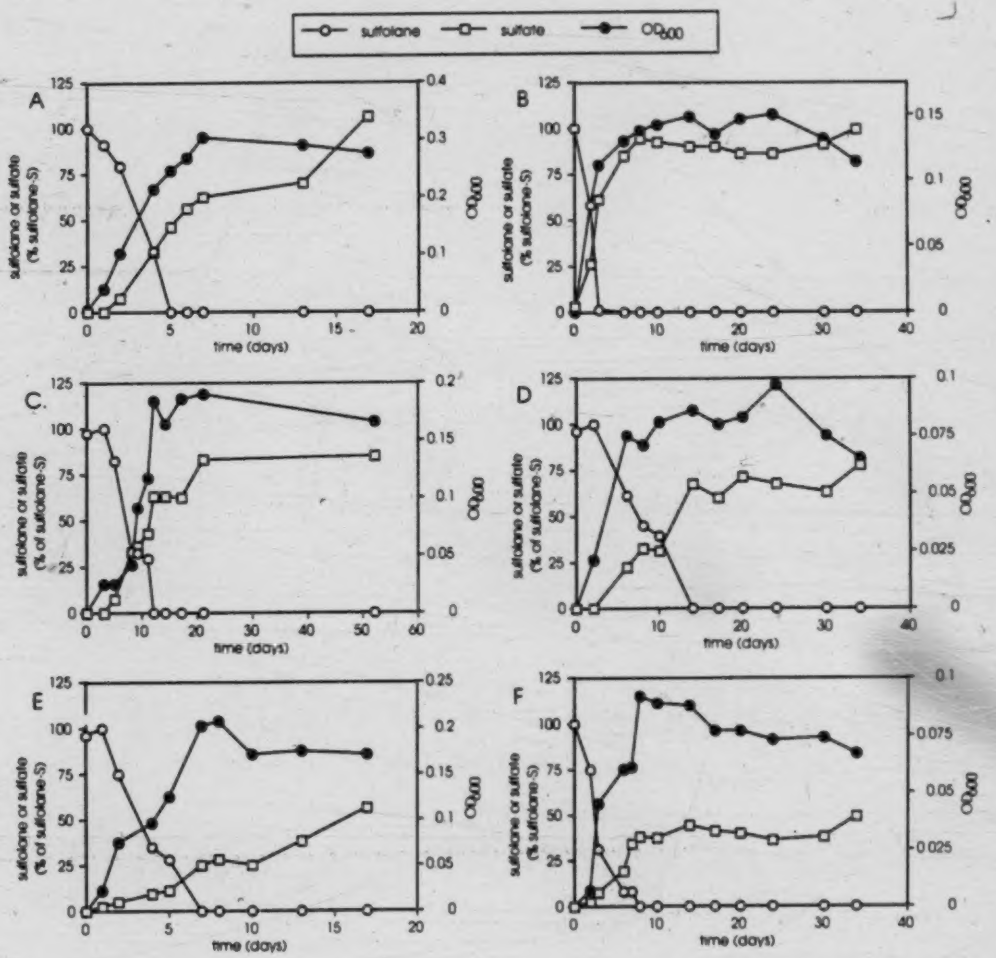


Figure 3.23. Release of $SO_4^{=}$ over time in pure or mixed cultures growing on sulfolane as a sole source of C and S. Replicate experiments were carried out with each culture. A and B. HM8-S28. C and D. HP1-S28. E and F. WP1-S28.

< 2 d. The immediate release of $SO_4^{=}$ in these cultures, when compared with the slight lag observed in the sulfolane-degrading cultures (Figure 3.23), may be due to the slower growth rate of the sulfolane degraders. Although sulfolane removal by the mixed culture occurred over 2 d, the other cultures that were incubated required 5 to 13 d to completely remove sulfolane from the culture supernatant, and the mixed culture was also incubated and monitored over this time.

In the serum bottle C and S balance experiments, complete conversion of sulfolane to $SO_4^{=}$ was not observed for the mixed culture HM8-S28; $SO_4^{=}$ recovery was 65% to 85% after a 30 d incubation. The incubation period ought to have been sufficient; complete release of $SO_4^{=}$ was observed after 17 to 34 d in the shake-flask incubations used for the $SO_4^{=}$ release experiments. It is possible that for the growth of this mixed culture, more O_2 was required than the amount calculated for sulfolane degradation. The amount of $SO_4^{=}$ released was higher at lower sulfolane concentrations in the serum bottle incubations (Table 3.23). In the mixed culture, many complex interactions between the members of the microbial community were likely to have occurred, including scavenging of dead cells or metabolic byproducts by heterotrophs unable to utilize sulfolane; these activities may have resulted in the depletion of O_2 to levels that interfered with the complete mineralization of sulfolane-S to $SO_4^{=}$. It is also possible that in conditions where O_2 depletion occurred, the decrease in available O_2 may have been sensed by the cells, triggering changes in cell metabolism which directed sulfolane-S to pathways that did not utilize O_2 or form $SO_4^{=}$. Further experimentation would be required to determine whether either of these possibilities occurred.

In contrast, the recovery of $SO_4^{=}$ in both pure cultures was similar for the serum bottle C and S balance experiments and the shake-flask time course experiments for $SO_4^{=}$ release from sulfolane. For HP1-S28, a slight difference was noted between incubation types; 50% to 78% of sulfolane-S was released as $SO_4^{=}$ in the serum bottles and 77% to 85% in the shake-flask cultures. However, given the variability in the serum bottle experiment (only one of four cultures showed the low value of 50%, the others ranged from 65% to 78%); this difference (from the 77 to 85% values for the shake-flask cultures) was not felt to be significant. For WP1-S28, $SO_4^{=}$ release was 39% to 55% in the serum bottles and 49% to 56% in the shake-flask cultures; these results were similar between both incubation types. Some S-containing compounds must have remained in the two pure cultures, or were released from them as volatile substances, while the mixed culture was able to completely mineralize sulfolane-S to $SO_4^{=}$. It was not possible to determine whether the undetected S-containing metabolites of sulfolane were associated with the sulfolane-derived soluble organic C detected in the culture supernatants (Table 3.23). Attempts were made to identify the C- and S-containing compounds remaining after sulfolane degradation (Appendix D).

3.9 Identification of the pure culture WPW-S28 by 16S rRNA sequence, physiological traits and morphology of cells and colonies

WPW-S28 was selected for identification because it was the pure culture most active against sulfolane. This bacterial culture also demonstrated the most reproducible results for growth on the compound.

3.9.1 Sequence of 16S rRNA and identification of WPW-S28 using its 16S rRNA sequence

Genomic DNA from WPW-S28 was isolated, and the 16S rRNA gene was amplified by PCR, then sequenced (Section 2.14.1). A previously identified environmental isolate, *Paenibacillus polymyxa*, was used as a control to ensure that the sequencing results were accurate. The *P. polymyxa* isolate had previously been identified using its physiological characteristics and fatty acid methyl ester composition (P. Beatty, personal communication). Sequencing and amplification primers were derived from Hauben et al. (1995) and are listed in Table 2.8. The primer sequences suggested by Hauben et al. (1995) were altered to ensure optimum annealing in the sequencing reactions. These alterations were based on sequences already obtained from WPW-S28 and *P. polymyxa* using the amplification primers for sequencing. The primer length was changed to yield a T_m of at least 55°C. The T_m of some primers suggested by Hauben et al. (1995) was too low for the available sequencing techniques.

The structure of 16S rRNA is complex; the entire molecule is involved in a series of stem loops. It is important for the ends of primers, particularly the 3' ends, not to be involved in stem loops as this type of structure can prevent the primers from binding to template DNA during the sequencing reactions. Nucleotide substitutions and additions to the primers of Hauben et al. (1995) were therefore made with reference to the folded structure of 16S rRNA given in Woese (1987). Primers were selected in regions of DNA where the sequences of *P. polymyxa* and WPW-S28 were similar; these were likely to be highly conserved regions. All sequencing was done in tandem for WPW-S28 and *P. polymyxa*; DNA amplification and purification was done in part by P. Beatty (Dept. of Biological Sciences, University of Alberta, Edmonton, AB). The sequence of the WPW-S28 rRNA gene is shown in Figure 3.24. Highly conserved bases that are used to distinguish eubacteria from archaebacteria (Woese, 1987) are marked above the WPW-S28 sequence in Figure 3.24. Only 19 of the WPW-S28 bases were different from the 569 conserved bases (Woese, 1987), representing a 3.3% divergence from the expected sequence. This low level of divergence suggested that the amplification of 16s rRNA gene from the WPW-S28 genome and subsequent sequencing introduced few errors in the nucleotide order. This sequence was submitted to the Basic Local Alignment Search Tool (BLAST) and the Ribosomal Database Project (RDP) to determine the identity of WPW-S28 by comparison with the 16s rRNA gene sequences of known microorganisms.

3.9.1.1 Identity of WPW-S28 from BLAST search

A BLAST search (Altschul et al., 1997; Peruski and Peruski, 1997) for the identities of DNA sequences most similar to the WPW-S28 16s rRNA gene sequence was carried out. The results of this search are summarized in Table 3.24. Only sequences that were over 1000 bases (the WPW-S28 sequence was 1353 bases) were included in this table. The sequence for WPW-S28 16s rRNA was most similar to *Variovorax paradoxus* 16s rRNA. Other similar 16s rRNA sequences included those for environmental isolates such as an unidentified 2,4-D-degrading bacterium, strain HW1 (Kamagata et al., 1997) and a trichloroethylene-degrading strain of *V. paradoxus* (Hamada and Harayama, unpublished data). *V. paradoxus* was formerly known as *Alcaligenes paradoxus* (Holt et al., 1994).

The name *Alcaligenes* means alkali-producing bacteria; these organisms release alkaline products during the metabolism of a number of organic compounds. The species name *paradoxus* refers to the metabolism of this group of bacteria; while most *Alcaligenes* species are chemoorganotrophs, one group of *A. paradoxus* are chemolithotrophs, aerobically oxidizing H_2 for energy (Kersters and De Lay, 1984). This group (Biovar I) is also able to reduce NO_3^- to NO_2^-, although they are obligate aerobes; the other group (Biovar II) is not able to do this (Kersters and De Lay, 1984). Willems et al. (1991) proposed that *A. paradoxus* be placed in a new genus, *Variovorax*, based on DNA hybridization studies with this organism and the *Alcaligenes* type species, *A. faecalis*. There is currently only the one species, *paradoxus*, in the genus *Variovorax*. The name *Variovorax* is derived from the words 'various' and 'voracious', which reflects the wide variety of substrates the organisms from this genus are capable of using (Willems et al., 1991).

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      .   •  •••.  ••     .• •  ••••. •••    •.         .         .                •••.•  • • •••••  o •
1     ATTGAACGCTGGCGGCATGCCTTACACATGCAAGTCGAACGGTAACGGCAGCGCGGGAGCTTTCCTGGCGACGAGTGGCGAACGGGTGAGTAATACCTGG 100
      . • ••  • .        •.••• •    .••••     . •••• •  .••       .      •• .         .         .        •
101   GAACCTGCCCAATCGAGGGGGATAACGCAGCGAAAGCTGTGCTAATACCGGAACCTGCCCAATCGAGGGGGATAACGCAGCGAAAGCTGTGCTAATACCG 150
      • o       •    •• ••.• •  •   . •••• •• .   •  • •.   •    ••   •   ••.•••   •  •    • •  •   ••••••
201   AGCGGCCCATGGCAGATTAGCTAGTTGGTGGGGTAACGGCTCACCAAGCCTTCGATCTGTAGCTGGTCTGAGAGGATGACCAGCCACACTGGGACTGAGA 300
      •• •   •  .  ••••••••  ••• ••o.   •••   •   •• •• . • ••    ••    o   . ••  ••• .         .
301   CACGGCCCAGACTCCTACGGGAGGCACCATTGGGGAATATTCCACAATGGGCGCAACCCTGATGCACCCATCCCCCGTGTTTAAAAAAGGCCTCCGGGTT 400
      .         .         .         .         .         .         .         •••    •• .••    •••.•••••••••
401   GTAAAGTACTTTCACCGGGGAGGAAGGGAGTAAATTTAATACCTTTGCTCATTGACGTTACCCGTAAGATAAGCACCGGCTAACTCCGTGCCAGCAGCCG 500
      •••••• •o .      • •• • •     .  • • ••••  ••••• •.  • ••  ••        •o        •••       •••  •••
501   CGGTAATAAGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTAATGTAAGACAGTTGTGAAATCCCCGGGCTTAACCTGG 600
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601   GAAGGGCATTTGAGACTGCATTGCTTGAGTACGGTAGAGGGGGGTGGAATTTCGTGTGTAGCAGTGAAATGCGTAGATATGCGGAGGAACACCGATGGCG 700
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701   AAGGCGATCCCCTGGGCCTGTACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACT 800
      .•        .         .     o o . ••  ••  .   •••   .•••••••••.••••••  ••••••  • ••••• •••••••••• ••••
801   TGGTTGTTGGGTCTTCACTGACTCAGTAACGAAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGG 900
      .•••••••••.••••••  ••••••  • ••••• •••••••••• ••••• •• •••  .         .         .         .
901   GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAAAACCTTACCCACCTTTGACATGTACGGAATTCGCCAGAGATGGCTTAGTGC 1000
      .         .         .   •••••••  • •• ••••••••   ••••   ••  .••• •••• .• ••o•••••  ••••   .     •••
1001  TCGAAAGAGAACCGTAACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAAAGAGCGCAACCCCTATCCTTTGTTG 1100
      .••       .     • ••.•       •o •••   • . •   •••••••••   ••.  •o•  • .•o   ••• •••  •    . ••••  ••
1101  CCAGCGGTCCGGCCGGGAACTCAAAGGAGAATGCCAGTGATAAACTGGAGGAAGGTGGGGAGGAAGTCAAGTTATTATGGCCCTTACGACCAGGGCTACA 1200
      •••• oo••••••••     •••    •  .• •    • .•      • .•• •    •••      • .•••   •••.•  • ••• ••• •• •
1201  CACGTCATACAATGGCTGGTACAAAGGGTTGCCACCCCGCGAGGCGGAGCTAATCCCATAAAATCAGTCGTAGTCCGGATCGCAGTCTGCAACTCGACTG 1300
      • ••••   ••• •   •••••• ••o   •••  •    o••• •••• .•• •• •  ••••• ••••••••••••
1301  CGTGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCG 1378
```

Figure 3.24. The 16s rDNA sequence for WPW-S28. Bases that are highly conserved among all eubacteria (Woese, 1987) are marked: closed circles above bases which match the WPW-S28 gene sequence, and open circles above bases where the WPW-S28 sequence did not match the highly conserved bases. Small dots mark every 10th base pair.

Table 3.24. BLAST search % similarity and bit scores for 16S rRNA sequence similarities between WPW-S28 and other microorganisms, and comparison to RDP results. All BLAST P values were 0.0.

organism identity from BLAST similarity search	% similarity	bits	organism identified in RDP search
Variovorax paradoxus	96	1043	yes
Variovorax paradoxus environmental isolate	96	1037	yes
Variovorax paradoxus MBIC3839	95	1011	yes
Unidentified bacteria 16S rRNA strain HW1	95	1003	yes
Variovorax sp. WFF52 DNA for 16S rRNA	95	995	no
Acidovorax sp. IMI 357678 16S ribosomal RNA	95	989	no
Bacteria species 16S rRNA gene, strain T3	95	987	yes
uncultured bacterium SJA-62 16S rRNA gene	95	987	no
Xylophilus ampelinus 16S ribosomal RNA gene	95	979	no
Aquaspirillum delicatum 16S ribosomal RNA	95	979	no
Acidovorax konjaci 16S ribosomal RNA gene	94	975	no
Acidovorax avenae subsp. avenae	94	955	no
Aquaspirillum gracile 16S ribosomal RNA gene	94	955	no
Pseudomonas lanceolata DNA for 16S rRNA	94	954	no
Acidovorax avenae subsp. citrulli	94	948	no
uncultured bacterium SJA-23 16S rRNA gene	94	948	no
Acidovorax avenae subsp. cattleyae	94	940	no
Beta proteobacterium B7 16S ribosomal RNA	94	940	yes
Acidovorax temperans 16S ribosomal RNA gene	94	940	no
Acidovorax avenae subsp. avenae 16S rRNA	94	938	no
Type 0803 filamentous bacterium 16S rRNA gene	94	934	yes
Acidovorax sp. 7078 16S ribosomal RNA gene	94	932	no
Acidovorax delafieldii 16S ribosomal RNA gene	94	932	no
Acidovorax facilis 16S ribosomal RNA gene	94	932	no
Hydrogenophaga palleronii 16S rRNA gene	94	932	yes
Hydrogenophaga sp. DSM 5680 16S rRNA gene	94	932	yes
Denitrifying Fe-oxidizing bacteria 16S rRNA	94	926	no

3.9.1.2 Identity of WPW-S28 from Ribosomal Database Project

The WPW-S28 16s rRNA sequence was submitted to the RDP to determine its relatedness to other bacteria and to construct a phylogenetic tree. The program was set so that only sequences ≥ 1000 bases in length were considered. The RDP uses a gapped sequence alignment protocol to identify the organisms with the most similar 16S rRNA sequence to the gene sequence submitted; it also calculates a similarity rating for each identity (Maidak et al., 1997). An exact sequence match would produce a similarity rating of 1. The similarity ratings for identities of the bacteria most closely related to WPW-S28 were as follows: *V. paradoxus* 0.664, *Comomonas testosteronii* 0.599, *Hydrogenophaga palleronii* 0.598 (data not shown). Organisms suggested by the RDP that were also suggested by BLAST are summarized in Table 3.24.

The sequence alignment for WPW-S28 and the *V. paradoxus* strain it most closely resembled is shown in Figure 3.25. The *V. paradoxus* strain that showed the strongest similarity to WPW-S28 was a metalotolerant organism (Anzai et al., unpublished data). The *Hydrogenophaga palleroni* strain that was similar to WPW-S28 was able to use 4-carboxy-4'-sulfoazobenzene as a sole source of C and energy. Two unidentified environmental isolates, strain T3 and strain T25, also showed similarity to WPW-S28. Both these isolates were included in a study to demonstrate the high genetic diversity found in a microbial community (Amann et al., 1996). Thus, WPW-S28 was similar to other environmental isolates, some of which were capable of degrading or tolerating xenobiotic compounds.

The RDP can be used to generate phylogenetic trees for different organisms, based on their 16S rRNA sequence similarity to organisms with 16S rRNA gene sequences already in the RDP database (Maidak et al., 1997). An unrooted phylogenetic tree for WPW-S28 was generated using this program (Figure 3.26). This tree shows a number of well-known organisms such as *E. coli* and *Pseudomonas fluorescens,* to give a frame of reference for WPW-S28. The tree was generated from members of the α, β and γ subgroups of the purple bacteria, which include most of the members of this division of eubacteria, and represents most of the known species of Gram-negative bacteria (Woese, 1987). The relatively long branch for WPW-S28 (Figure 3.26) is indicative of its imperfect match with *V. paradoxus*. This imperfect match demonstrated the importance of using the physiological and morphological characteristics of WPW-S28 to confirm its identity.

```
                 .          .         .         .        |         .
V.paradoxus AUUGAACGCUGGCGGCAUGCCUUACACAUGCAAGUCGAACGG------CAGCGCGGGAGC   54
            ..........................................●●●●●●............
WPW-S28     AUUGAACGCUGGCGGCAUGCCUUACACAUGCAAGUCGAACGGUAACGGCAGCGCGGGAGC   60
                     .         .         .         .         |         .

                 .         .         .         .         |         .
V.paradoxus AAUCCUGGCGGCGAGUGGCGAACGGGUGAGUAAUACAUCGGAACGUGCCCAAUCGUGGGG  114
            **..................................+.+.....+..........+....
WPW-S28     UUUCCUGGCGACGAGUGGCGAACGGGUGAGUAAUACCUGGGAACCUGCCCAAUCGAGGGG  120
                     .         .         .         |         .         .

                 .         .         .         |         .         .
V.paradoxus GAUAACGCAGCGAGNGCUGUGCUAAUNCCGCAUACGAUCUACGGAUGAAAGCAGGGGAUC  174
            .............++...........+.......+................++.....+.
WPW-S28     GAUAACGCAGCGAAAGCUGUGCUAAUACCGCAUAAGAUCUACGGAUGAAAGAGGGGGACC  180
                     .         .         |         .         .         .

                 .         .         |         .         .         .
V.paradoxus GCAAGACCUUGCGCGAAUGGAGCGGCCGAUGGCAGAUUAGGUAGUUGGUGAGGUAAAGGC  234
            ..............+..+.........+......................+.....+...
WPW-S28     GCAAGACCUUGCGCCAACGGAGCGGCCCAUGGCAGAUUAGCUAGUUGGUGGGGUAACGGC  240
                     .         |         .         .         .         .

                 .         |         .         .         .         .
V.paradoxus UCACCAAGCCUUCGAUCUGUAGCUGGUCUGAGAGGACGACCAGCCACACUGGGACUGAGA  294
            ....................................+.......................
WPW-S28     UCACCAAGCCUUCGAUCUGUAGCUGGUCUGAGAGGAUGACCAGCCACACUGGGACUGAGA  300
                     |         .         .         .         .         |

                 |         .         .         .         .         |
V.paradoxus CACGGCCCAGACUCCUACGGGAGGCAGCAGUGGGGAAUUUUGGACAAUGGGCGAAAGCCU  354
            ..........................+...........+..++..........+..+...
WPW-S28     CACGGCCCAGACUCCUACGGGAGGCACCAUUGGGGAAUAUUCCACAAUGGGCGCAACCCU  360
                     .         .         .         .         |         .

                 .         .         .         .         |         .
V.paradoxus GAUCCAGCCAUGCCGCGUGCAGGAUGAAGGCCUUCGGGUUGUAAACUGCUUUUGUACGGA  414
            ...+..+....+..+....++++.++...................+++++++++++++++
WPW-S28     GAUGCACCCAUCCCCCGUGUUUAAAAAAGGCCUCCGGGUUGUAAAGUACUUUCACCGGGG  420
                     .         .         .         |         .         .

                 .         .         .         |          .         .
V.paradoxus ACGAAACGGCCUUUUCUAAUAAAGAGGGCUAAUGACGGUA-CCGUAAGAAUAAGCACCGG  473
            ++++++++++++++++++++++++++++++++++++++++●.......+...........
WPW-S28     AGGAAGGGAGUAAAUUUAAUACCUUUGCUCAUUGACGUUACCCGUAAG-AUAAGCACCGG  479
                     .         .         |         .          .

                  .         .         |         .         .         .
V.paradoxus CUAACUACGUGCCAGCAGCCGCGGUAAUACGUAGGGUGCAAGCGUUAAUCGGAAUUACUG  533
            ......+......................+..............................
WPW-S28     CUAACUCCGUGCCAGCAGCCGCGGUAAUAAGUAGGGUGCAAGCGUUAAUCGGAAUUACUG  539
            .         .         |         .         .         .

                  .         |         .         .         .         .
V.paradoxus GGCGUAAAGCGUGCGCAGGCGGUAAUGUAAGACAGUUGUGAAAUCCCCGGGCUCAACCUG  593
            .....................................................+......
WPW-S28     GGCGUAAAGCGUGCGCAGGCGGUAAUGUAAGACAGUUGUGAAAUCCCCGGGCUUAACCUG  599
            .         |         .         .         .         .

                  |         .         .         .         .         |
V.paradoxus GGAACUGCAUCUGUGACUGCAUUGCUGGAGUACGGCAGAGGGGGAUGGAAUUCCGCGUGU  653
            ....++....+..+............+.................+.......+..+....
WPW-S28     GGAAGGGCAUUUGAGACUGCAUUGCUUGAGUACGGUAGAGGGGGGUGGAAUUUCGUGUGU  659
            |         .         .         .         .         |

                  .         .         .         .         |         .
V.paradoxus AGCAGUGAAAUGCGUAGAUAUGCGGAGGAACACCGAUGGCGAAGGCAAUCCCCUGGGCCU  713
            ..............................................+.............
WPW-S28     AGCAGUGAAAUGCGUAGAUAUGCGGAGGAACACCGAUGGCGAAGGCGAUCCCCUGGGCCU  719
            .         .         .         .         |         .

                  .         .         .         |         .         .
V.paradoxus GUACUGACGCUCAUGCACGAAAGCGUGGGGAGCAAACAGGAUUAGAUACCCUGGUAGUCC  773
            ............................................................
WPW-S28     GUACUGACGCUCAUGCACGAAAGCGUGGGGAGCAAACAGGAUUAGAUACCCUGGUAGUCC  779
            .         .         .         |         .         .

                  .         .          |         .         .         .
V.paradoxus ACGCCCUAAACGAUGUCAACU-GGUUGUUGGGUCUUNACUGACUCAGUAACGAAGCUAAC  832
            .....................●..............+.......................
WPW-S28     ACGCCCUAAACGAUGUCAACUUGGUUGUUGGGUCUUCACUGACUCAGUAACGAAGCUAAC  839
            .         .         |         .         .

                   .         |         .         .         .         .
V.paradoxus GCGUGAAGUUGACCGCCUGGGGAGUACGGCCGCAAGGUUGAAACUCAAAGGAAUUGACGG  892
            ............................................................
WPW-S28     GCGUGAAGUUGACCGCCUGGGGAGUACGGCCGCAAGGUUGAAACUCAAAGGAAUUGACGG  899
            .         |         .         .         .         .

                   |         .         .         .         .         |
V.paradoxus GGACCCGCACAAGCGGUGGAUGAUGUGGUUUAAUUCGAUGCAACGCGAAAAACCUUACCC  952
            ..+.................++......................................
WPW-S28     GGGCCCGCACAAGCGGUGGAGCAUGUGGUUUAAUUCGAUGCAACGCGAAAAACCUUACCC  959
            |         .         .         .         .         |
                   .         .         .         .         |         .
V.paradoxus ACCUUUGACAUGUACGGAAUUCGCCAGAGAUGGCUUAGUGCUCGAAAGAGAACCGUAACA   1012
            ------------------------------------------------------------
WPW-S28     ACCUUUGACAUGUACGGAAUUCGCCAGAGAUGGCUUAGUGCUCGAAAGAGAACCGUAACA   1019
            .         .         .         .         |         .

                   .         .         .         |         .         .
V.paradoxus CAGGUGCUGCAUGGCUGUCGUCAGCUCGUGUCGUGAGAUGUUGGGUUAAGUCCCGCAACG   1072
            ●●-----------------------------+----+-----------------------
WPW-S28     --GGUGCUGCAUGGCUGUCGUCAGCUCGUGUUGUGAAAUGUUGGGUUAAGUCCCGCAAAG   1077
              .         .         |         .         .         .

                   .         .         |         .           .         .
V.paradoxus AGCGCAACCCUUGUCAUUAGUUGCUACAUUUAGUUGGGC--ACUCUAAUGAGACUGCCGG   1130
            ----------+++++++++++++++++++++++++++++●●----+--+----+----+-
WPW-S28     AGCGCAACCCCUAUCCUUUGUUGCCAGCGGUCCGGCCGGGAACUCAAAGGAGAAUGCCAG   1137
              .         .         |         .         .         .

                     .         |         .         .         .         .
V.paradoxus UGACAAACCGGAGGAAGGUGGGGAUGACGUCAAGUCCUCAUGGCCCUUAUAGGUGGGGCU   1190
            ---+----+---------------+--+-------++-+----------++++++-----
WPW-S28     UGAUAAACUGGAGGAAGGUGGGGAGGAAGUCAAGUUAUUAUGGCCCUUACGACCAGGGCU   1197
              .         |         .         .         .         .

                     |         .         .         .         .         |
V.paradoxus ACACACGUCAUACAAUGGCUGGUACAAAGGGUUGCCAACCCGCGAGGGGGAGCUAAUCCC   1250
            -------------------------------------+---------+------------
WPW-S28     ACACACGUCAUACAAUGGCUGGUACAAAGGGUUGCCACCCCGCGAGGCGGAGCUAAUCCC   1257
              |         .         .         .         .         |

                     .         .         .         .         |         .
V.paradoxus AUAAAACCAGUCGUAGUCCGGAUCGCAGUCUGCAACUCGACUGCGUGAAGUCGGAAUCGC   1310
            ------+-----------------------------------------------------
WPW-S28     AUAAAAUCAGUCGUAGUCCGGAUCGCAGUCUGCAACUCGACUGCGUGAAGUCGGAAUCGC   1317
              .         .         .         .         |         .

                     .         .         .         |         .         .
V.paradoxus UAGUAAUCGUGGAUCAGAAUGUCACGGUGAAUACGUUCCCGGGUCUUGUACACACCGCCC   1370
            -------------------------------------------+----------------
WPW-S28     UAGUAAUCGUGGAUCAGAAUGUCACGGUGAAUACGUUCCCGGGCCUUGUACACACCGCCC   1377
              .         .         .         |         .         .
```

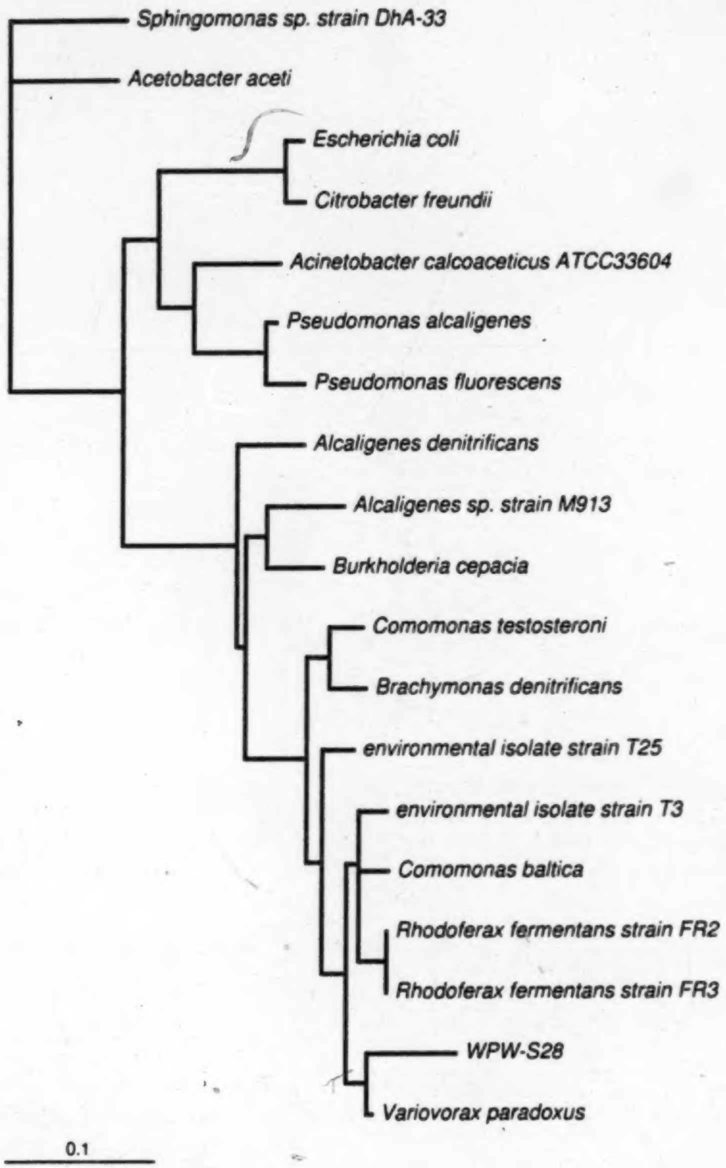
Sphingomonas sp. strain DhA-33
Acetobacter aceti
Escherichia coli
Citrobacter freundii
Acinetobacter calcoaceticus ATCC33604
Pseudomonas alcaligenes
Pseudomonas fluorescens
Alcaligenes denitrificans
Alcaligenes sp. strain M913
Burkholderia cepacia
Comomonas testosteroni
Brachymonas denitrificans
environmental isolate strain T25
environmental isolate strain T3
Comomonas baltica
Rhodoferax fermentans strain FR2
Rhodoferax fermentans strain FR3
WPW-S28
Variovorax paradoxus
0.1

Figure 3.26. A phylogenetic tree for WPW-S28. The bar is an indicator of Euclidian distance. This tree was obtained using the RDP (Maidak et al., 1997) and interpreted on the program Treeview.

3.9.2 Physiological tests for identification of WPW-S28

The pure isolate WPW-S28 was tested for its ability to grow on different media or utilize different substrates, to complement the 16S rRNA sequence results, which suggested that WPW-S28 was related to *V. paradoxus*. *V. paradoxus* is the only member of its genus (Holt et al., 1994), therefore identification of the organism at the genus level automatically places it in this species, or as a member of a new species of this genus.

3.9.2.1 API 20E and API 20NE identification tests

The simplest method of identifying WPW-S28 by biochemical means was to use the API 20E and API 20NE identification test kits (Section 2.14.2.1). These kits were designed for identification of Gram negative bacilli. The API 20E kit was unlikely to give a useful identification for WPW-S28; this test was developed to identify enteric bacteria. However, the biochemical tests in both kits helped to define the substrate utilization abilities of the organism, as well as providing a tentative identity. The tentative identity was useful for narrowing down the search for the closest related genus to WPW-S28. The API test results for WPW-S28 are summarized in Table 3.25.

The results from the API E and API NE tests for WPW-S28 were inconclusive; no pattern of substrate utilization corresponded to any organism listed in either reference manual. However, the pattern for WPW-S28 fell within a large section of *Pseudomonas fluorescens* using the API 20E test, and between *Listonella damsela* and *Pasteurella pneumotropica* with the API 20NE test. Many Gram-negative environmental isolates capable of degrading xenobiotic chemicals are *Pseudomonas* species (Cerniglia, 1984; Floodgate, 1995; Leahy and Colwell, 1990). Some *Listonella* species were observed in the β group of purple bacteria in the complete prokaryotic 16S rRNA phylogenetic tree at the RDP database web site, however this genus was quite distant from the organisms indicated to be closely related to WPW-S28 by this method. A *Pasteurella* species was reported to be in the γ subgroup (Woese, 1987), therefore this organism was also unlikely to be closely related to WPW-S28. According to Woese (1987), *Pseudomonas testosteronii, P. acidovorans* and *P. cepacia* (now *Burkholderia cepacia*) all belong to the β subgroup of purple bacteria, therefore WPW-S28 was more likely to be related to members of the genus *Pseudomonas* than to any other organisms suggested by the API tests.

Table 3.25. API E and API NE test results for WPW-S28. A + (positive) result for a substrate indicates utilization of the substrate within 48 h of inoculation in the test strip.

API 20E test		API 20NE test	
substrate, enzyme or test	+ or - result	substrate or test	+ or - result
β-galactosidase activity	-	nitrate reduction	+
arginine dihydrolase	+	tryptophane metabolism	-
lysine decarboxylase	-	glucose	+
ornithine decarboxylase	-	arginine dihydrolase	+
citrate	-	urease	+
H_2S from thiosulfate	-	esculin hydrolysis	-
urease	+	gelatin hydrolysis (protease)	-
tryptophane deaminase	-	β-galactosidase	-
indole from tryptophane	-	glucose	+
acetoin from glucose	+	arabinose	+
gelatin hydrolysis (protease)	-	mannose	weak+
glucose	+	mannitol	+
mannitol	+	N-acetyl-glucosamine	-
inositol	-	maltose	-
sorbitol	-	gluconate	+
rhamnose	-	caprate	-
sucrose	-	adipate	+
melibiose	-	malate	+
amygdalin	-	citrate	-
arabinose	+	phenylacetate	-
cytochrome oxidase	weak +	cytochrome oxidase	weak +

3.9.2.2 Growth of WPW-S28 on selective and differential media

Selective and differential media (described in Section 2.14.2.2) were used to assist in the identification of WPW-S28 (Table 3.26). This organism grew on *Pseudomonas P and F* agar but was not fluorescent under UV light, suggesting that it was not *P. fluorescens* as indicated by the API 20E test.

Results from partial 16S rRNA sequencing suggested that WPW-S28 might have been related to the genus *Salmonella* (data not shown). Inoculation of WPW-S28 on XLD agar, SS agar, Simmon's citrate agar, MacConkey agar and EMB agar demonstrated that it was not a fecal coliform, particularly not *Salmonella* or *Shigella*, however the type of growth observed on EMB agar was similar to that demonstrated by members of the family *Enterobacteriaceae*. WPW-S28 showed no hemolysis on blood agar plates. These results supported the evidence from 16S rRNA sequencing that WPW-S28 was not a *Pasteurella* species (Section 3.9.1) as suggested by the API 20NE test; this genus encompasses a group of pathogenic microorganisms, most of which show hemolysis on blood agar (Kersters and De Lay, 1984).

Mixed results were obtained for growth on sucrose. WPW-S28 probably did not utilize sucrose, but may have been able to grow on sucrose agar plates, using impurities in the medium or compounds carried over during its transfer. WPW-S28 also did not use lactose, dextrose or other complex sugars. Overall, WPW-S28 was capable of utilizing most monosaccharides that were tested, but no disaccharides (Table 3.26). Amygdalin, a cyanohydrin made up of two molecules similar in structure to monosaccharides (Fessenden and Fessenden, 1986), was also not utilized. All the monosaccharides tested except rhamnose (6-deoxymannose; Fessenden and Fessenden, 1986) have oxygen at the 6-position; this may have been the important factor for utilization of these compounds, since both 5-C (arabinose) and 6-C (glucose, mannose) sugars were utilized by WPW-S28. The difference between sorbitol, inositol and mannitol utilization was inexplicable; all were 6-C compounds with groups at each C, however only mannitol was used. In the case of other substrates, WPW-S28 favored shorter chain fatty acids. The organism grew well on acetate, succinate, malate and adipate and gave mixed results on citrate (all ≤ 6 C in length), however it was unable to use caprate (a 10 C compound) or phenylacetate.

Generally, WPW-S28 appeared to be able to use small compounds such as simple sugars, fatty acids ≤ 6 C in length, amino acids, and glycerol (Table 3.26) as well as sulfolane, while the more complex compounds such as disaccharides, starch, and

Table 3.26. Growth of WPW-S28 on different types of media. Where only a substrate name is indicated, agar plates consisting of B+N8P medium with the substrate as a sole C source and bromthymol blue as a pH indicator were used.

medium	growth, reaction or identifying characteristics
acetate	growth, basic products (identified by pH indicator in medium)
bile esculin	poor, no color change therefore not inhibited by bile but unable to hydrolize esculin
blood agar	good growth but no hemolysis was observed
catalase test	weakly positive
citrate	growth, basic products
EMB	pink mucoid colonies; not *Salmonella* or *Shigella* but may be a member of the family *Enterobacteriaceae*
glucose	growth, acidic products
glycerol	growth, acidic products
lactose broth	little growth, no gas production
Luria-Bertani agar	growth, formation of yellow, creamy, spindle shaped colonies
MacConkey agar	very poor growth, small sticky colonies, dye may be inhibitory although WPW-S28 is Gram negative
mannitol salt agar	no growth
Pseudomonas P and F agar	good growth, no fluorescence under UV light
SS agar	no growth; not likely to be *Salmonella, Shigella* or other enteric pathogenic Gram-negative bacillus
Simmons citrate agar	very poor growth, may use citrate but may not use NH_4^+ as a sole N source (mineral medium contains both NO_3 and NH_4^+); not a fecal coliform
starch agar	little or no starch hydrolysis observed
succinate	growth, basic products
sucrose	growth, acidic products
triple sugar iron slant	good growth, yellow (no acidic products or FeS precipitate); utilized protein but not lactose, dextrose or sucrose, no thiosulfate reduction to sulfide
XLD agar	no growth; unlikely to be an enteric bacterium

hydroxyl longer chain fatty acids were not used. Some smaller compounds such as rhamnose, sorbitol and iniositol also were not used by WPW-S28; possibly due to enzyme specificity as they were similar in structure to compounds used by WPW-S28.

Table 3.27 summarizes some physiological traits of *V. paradoxus*. Only one biochemical test provided different results for WPW-S28 than were published for *V. paradoxus*; WPW-S28 grew poorly on citrate, while ≥ 90% of *V. paradoxus* strains grew on this compound (Kersters and De Lay, 1984). These observations are in agreement with the inability of WPW-S28 to utilize more carbohydrates than simple sugars, and its metabolism of fatty acids, an amino acid and glycerol (Sections 3.9.2.1 and 3.9.2.2).

3.9.2.3 Colony morphology and cell stains of WPW-S28

WPW-S28 grew on rich medium such as LB agar as creamy, yellow, shiny, opaque colonies that were 3 to 5 mm in diameter. Colonies were smooth, with an entire margin, elongated in a spindle manner and umbonate, with the raised central portion elongated along the central axis. When growing on rich medium, the organism did not produce any unusual odor, however growth in the presence of sulfolane produced a strong sulfurous odor, whether in liquid or on solid medium. This organism appeared to make polysaccharide; colonies were slimy and glistening, and became extremely sticky over time. *V. paradoxus* generally produces yellow, mucoid colonies on rich agar (Busse and Auling, 1992; Holt et al., 1994; Kersters and De Lay, 1984).

Under the microscope WPW-S28 cells were Gram negative bacilli with a 3:1 length:width ratio and truncate or slightly rounded ends. Cells appeared to be single; no groups were observed in cultures grown on liquid or solid medium. The cells were smaller than *E. coli* and approximately half the size of *P. polymyxa*. *E. coli* are approximately 2.0 to 6.0 μm long and 1.1 to 1.5 μm in diameter; *V. paradoxus* are approximately 0.5 to 2.6 μm long and 0.5 to 1.0 μm wide (Holt et al., 1994; Kersters and De Lay, 1984). WPW-S28 cells were therefore a similar size to *V. paradoxus* cells. No spores or endospores were observed under any conditions, even in cultures that had been incubated for more than a month.

A modified Gray flagellar stain (Section 2.14.2.3, Mayfield and Innis, 1977) detected no flagella on WPW-S28, which was reasonable because live cultures did not appear to be motile, and no motility was observed in motility agar. *P. polymyxa* was used as a positive control for the flagellar staining method; peritrichous flagella were easily visible on these cells, which were previously known to be motile and to have this type of flagella. It was therefore concluded that WPW-S28 was not motile. *V. paradoxus* is

Table 3.27. Comparison between physiological characteristics of *Variovorax (Alcaligenes) paradoxus* and the same traits observed for isolate WPW-S28. Information from Holt et al. (1994), Kersters and De Lay (1984) and Busse and Auling (1992).

characteristic or nutrient used	% of *Variovorax paradoxus* strains positivefor test	WPW-S28
acetate	≥ 90	+
adipate	11 to 89	+
arabinose	≥ 90	+
caprate	≤ 10	-
catalase	≥ 90	weak +
citrate	≥ 90	± (both + and – results)
dextrose	≤ 10	-
gelatin hydrolysis	11 to 89	-
gluconate	≥ 90	+
glucose	≥ 90	+
glycerol	≥ 90	+
inositol	≤ 10	-
lactose	≤ 10	-
malate	11 to 89	+
maltose	≤ 10	-
mannitol	≥ 90	+
mannose	≥ 90	+
NO_3^- reduction to NO_2^-	≥ 90	+
oxidase	≥ 90	weak +
phenylacetate	11 to 89	-
rhamnose	11 to 89	-
sorbitol	≤ 10	-
starch	≤ 10	-
sucrose	≤ 10	-
tryptophane	≤ 10	-
urease	≥ 90	+

motile, and has two to four degenerate peritrichous flagella (Holt et al., 1994; Kersters and De Lay, 1984), however these flagella are fine in structure (Kersters and De Lay, 1984). If WPW-S28 had delicate flagella, they may have been disrupted during the staining process and therefore not observed.

3.9.3 Identity of WPW-S28, based on the 16S rRNA sequence, cell and colony morphology and biochemical tests

The 16S rRNA sequence data generated for WPW-S28 suggested that this organism was related to *V. paradoxus*, formerly known as *A. paradoxus*. This identity was not an excellent match, however biochemical evidence, and colony and cellular morphology, supported the identity of WPW-S28 with *V. paradoxus*. The greatest difference between WPW-S28 and *V. paradoxus* was the lack of motility of WPW-S28. The remaining evidence was strongly in favor of WPW-S28 being a member of the genus *Variovorax*, and possibly of the only species currently defined in that genus, *paradoxus*. If WPW-S28 does belong to the species *paradoxus*, its ability to reduce NO_3^- (Section 3.9.2.1) suggests that it is probably a member of Biovar I (Kersters and De Lay, 1984). However, its low similarity to the type organism suggests that it may represent an as-yet undescribed species in the genus *Variovorax*.

V. paradoxus strains isolated from various contaminated sites have been demonstrated to degrade xenobiotic compounds. Di Giovanni et al. (1996) discovered 32 isolates capable of degrading 2,4-D. These isolates contained catabolic plasmids that varied in size and genetic content, suggesting that frequent gene rearrangements occurred in this group of organisms. Kamagata et al. (1997) detected a number of 2,4-D-degrading strains of *V. paradoxus* in a pristine soil. Mergaert et al (1993) observed *V. paradoxus* strains capable of degrading poly-(3-hydroxybutyrate) and poly-(3-hydroxybutyrate-co-3-hydroxyvalerate). These isolates and the strains previously mentioned (Sections 3.9.1.1 and 3.9.1.2) were all able to degrade xenobiotic compounds. Since *V. paradoxus* has been demonstrated to degrade a number of xenobiotic compounds, it is not surprising to find that a bacterial isolate capable of biodegrading sulfolane might belong to this genus, and possibly this species.

4 SUMMARY, CONCLUSIONS AND DIRECTIONS FOR FUTURE RESEARCH

Previous research has demonstrated that aerobic sulfolane degradation can occur, and that it is positively affected by nutrient amendment. This thesis research was done to obtain a greater understanding of sulfolane biodegradation. The research was divided into two general categories. The first was determination of the sulfolane degradation potential at specific contaminated sites. Sulfolane-degrading populations were detected in microcosms inoculated with site materials, and their nutrient requirements were assessed. The second focused on contributing to the general knowledge regarding sulfolane biodegradation, particularly the extent of mineralization of the compound by microbial populations and the types of bacteria able to degrade sulfolane. In order to address this task, a medium to detect sulfolane-degrading organisms was developed. The ability of sulfolane-degrading bacteria to convert the compound to CO_2, $SO_4^{=}$ and biomass was measured, and one vigorously active pure isolate was identified.

4.1 Laboratory research involving aquifer sediments and soils

Three projects using aquifer sediment or wetlands samples from Sulfinol® plant sites were undertaken to define the microbial activity against sulfolane. The first project was to detect aerobic and anaerobic sulfolane biodegradation using aquifer sediment samples from three contaminated sites. Aerobic activity against sulfolane was detected at all three sites. However, the dissolved O_2 present in sulfolane-contaminated aquifers was negligible (J. Sevigny, Komex International, Calgary, AB; personal communication). Anaerobic sulfolane biodegradation occurred in one of two NO_3^--reducing and two Mn(IV)-reducing microcosms from site 1 and one Mn(IV)-reducing microcosm from site 3. NO_3^--, Mn(IV)-, and Fe(III)-reducing organisms were detected at all three sites, $SO_4^{=}$-reducing bacteria at sites 2 and 3, and methanogenic bacteria at site 1, however only three of these bacterial populations degraded sulfolane. This information, combined with the known migration of the compound and the dissolved O_2 concentration in the aquifers and groundwaters at the three sites, suggested that anaerobic sulfolane biodegradation played a minimal role in its attenuation at contaminated sites.

The second and third projects focused on the nutrient requirements for aerobic sulfolane biodegradation at site 2. At this site, sulfolane has migrated from landfill cells through an aquifer, and into two wetlands regions (Figure 2.1). The sediments and wetlands contaminated with sulfolane from the landfill cells, and an uncontaminated sediment, were studied in shake-flask cultures, respirometer microcosms (Salanitro et al., 1996; 1997) and 2.5-L microcosms (Holm et al., 1992). Sulfolane is also present in sediments near the Sulfinol® plant buildings at this site; this sediment was used in air-sparged microcosms (Fedorak and Coy, 1996; Mueller et al., 1991).

The microbial communities from the wetlands regions degraded sulfolane after a short lag time. In these samples, the settled layer appeared to be anaerobic, although the overlying aqueous layer contained 7 to 9 mg/L dissolved O_2. Pristine aquifer sediment samples were able to develop a sulfolane-degrading population over time if mixing and low levels of dissolved O_2 (2 mg/L or more) were provided, but in the absence of mixing no sulfolane degradation was observed. Contaminated aquifer sediment populations were able to degrade sulfolane. In almost all cases, the addition of N and P stimulated sulfolane biodegradation. Interestingly, the east and west plume contaminated aquifer populations appeared to require P supplementation to stimulate activity against sulfolane, while the subsurface sediments taken from near a Sulfinol® plant building required N supplementation to stimulate the same activity. For all of the contaminated sediment samples, sulfolane biodegradation was eventually observed. Supplementation with the more important nutrient increased the biodegradation and decreased the lag time before biodegradation commenced; the addition of a second nutrient frequently improved sulfolane biodegradation further, although this nutrient generally had little effect on biodegradation when added alone.

The overall interpretation of these results is that mixing and aeration of the contaminated sediment or groundwater materials was the most important factor for stimulating sulfolane biodegradation by their microbial populations. Aeration likely played an important role in this activity; previous experiments demonstrated that most microbial activity against sulfolane was aerobic. If O_2 was provided to the cultures or microcosms, further stimulation of biodegradation could be attained by supplementing them with inorganic sources of N and P. In all cases, the addition of both nutrients was more effective for stimulating biodegradation than addition of only one. The most limiting nutrient varied, depending on the sample location. Similar results with respect to the stimulation of biodegradation by the addition of N and P sources were observed by Braddock et al. (1997), Mueller et al. (1991) and Piehler and Paerl (1996).

Microcosm experiments were done using the 2.5-L incubation bottle method described by Holm et al. (1992), Nielsen and Christensen (1994), and Nielsen et al.

(1995a; 1995b). Nielsen and Christensen (1995a; 1995b) demonstrated that *in situ* and laboratory incubations of 2.5-L microcosms resulted in similar amounts of biodegradation for xenobiotic compounds. Holm et al. (1992) observed more biodegradation in some *in situ* microcosms than in the laboratory incubations. Nielsen and Christensen (1994) demonstrated that biodegradative activity was frequently similar in samples taken from a number of sites in the same hydrogeologic region, however samples taken from a different hydrogeologic region at the same site demonstrated different biodegradative abilities. The samples were all taken quite close together, however one aquifer sediment had a different groundwater source than the others (Nielsen and Christensen, 1994).

Similarly, groundwater that had passed through different landfills at site 2 contained different concentrations of sulfolane, DIPA and total organic C (Table 2.2), and samples from each location demonstrated different microbial activities against sulfolane. However, the aquifer sediment populations taken from both contaminated plumes showed a greater requirement for P than for N supplementation, suggesting that the entire region had the same nutrient requirements in spite of some differences in contaminant concentrations in each plume. Both observations were reasonable as the groundwater source was the same for both contaminated plumes, however by passing through different landfill areas, the groundwater was probably contaminated with different compounds, which would have affected the development of microbial populations in each plume differently. Once sulfolane reached the edge of the wetlands regions, it was depleted to very low concentrations in the surface water, either through biodegradation, dilution, or a combination of both. It is unlikely that sulfolane interacted with wetland solids; Luther et al. (1998) demonstrated that sulfolane and clay minerals had minimal interactions, while virtually no interaction occurred between sulfolane and soil organic matter.

The overall implications of this research are as follows.

1. No significant attenuation of sulfolane occurred at site 2; the compound spread freely through the aquifer supplied by groundwater that passed through the landfill areas. Detailed observations were not made for sites 1 and 3. However, given the groundwater sulfolane concentrations in contaminated regions at these sites, it is unlikely that sulfolane attenuation has been significant in these areas.
2. Sulfolane attenuation or dilution to undetectable levels likely occurred in the wetlands downgradient from the contaminated aquifers. If sulfolane were not diluted in these regions, microbial populations active against this compound under conditions of low

mixing and limited O_2 availability were demonstrated to be present in the wetlands region, therefore sulfolane attenuation may have occurred in these regions.

3. Sulfolane-degrading populations could develop in previously uncontaminated aquifer materials, however they required both mixing and aeration in order to develop within a period of approximately 1 y. Supplementation with inorganic N and P did not stimulate the development of this population in the absence of mixing. Presumably the development of a sulfolane-degrading bacterial population in a pristine aquifer is possible; sulfolane-contaminated aquifer sediments did contain a sulfolane-degrading population, which must have developed over time in these regions.
4. Sulfolane-degrading populations were present in all the contaminated aquifer sediments examined. Anaerobic populations were limited, and only appeared under NO_3^-- and Mn(IV)-reducing conditions, although degradation of the compound was proven to be thermodynamically feasible under all the anaerobic conditions tested, therefore aerobic biodegradation of sulfolane is likely to be the only way significant attenuation might be achieved in a contaminated aquifer.
5. Aerobic sulfolane biodegradation was stimulated by the addition of inorganic N and P sources. The addition of both nutrients provided the best stimulation of biodegradative activity. The relative importance of each nutrient varied depending on the sampling location.
6. Sulfolane biodegradation rates were determined in aquifer-like laboratory microcosms inoculated with material from site 2. No sulfolane biodegradation occurred in 2.5-L microcosms consisting of east and west plume aquifer materials from site 2 in the absence of added P. With added P, biodegradation occurred at 0.5 mg/L/h in the east plume microcosm and at 0.3 mg/L/h in the west plume microcosm (Section 3.3.3). Air-sparged microcosms inoculated with material from the Sulfinol® building location at site 2 degraded sulfolane at 0.4 mg/L/h (Section 3.5.3.2) in the absence of added nutrients other than O_2. Overall, these results suggest that with the appropriate modifications, such as the addition of air and inorganic nutrients, sulfolane biodegradation is possible in Sulfinol®-contaminated regions at site 2. If these biodegradation rates are used in any groundwater modeling, it is important to keep in mind that the rates were determined under laboratory conditions, and would likely be much lower in the field.

4.2 Properties of sulfolane biodegradation by defined bacterial cultures and the identity of a sulfolane-degrading bacterial culture

In order to isolate sulfolane-degrading bacteria for metabolic studies, a differential medium to detect sulfolane degraders, PAT, was developed. This medium was also useful for detecting and monitoring sulfolane-degrading bacterial populations in site-oriented projects. This medium had a 93% accuracy rate for distinguishing between degraders and nondegraders.

Once sulfolane-degrading organisms had been isolated, they were used in experiments designed to develop a better understanding of the processes involved in sulfolane biodegradation. All the organisms tested were able to grow on sulfolane, releasing $H_2SO_4^{=}$. Once cultures had reached pH 3.5 to 4.0, no further microbial activity was detected. Problems with pH drop were less likely to occur *in situ*, where the acid produced by sulfolane biodegradation could be employed in abiotic reactions or by bacterial populations, diluted by groundwater or neutralized by the presence of calcareous clays. Cultures inoculated with wetland materials demonstrated the ability to degrade sulfolane, even at low pH.

Sulfolane mineralization to CO_2, $SO_4^{=}$ and conversion to biomass by pure bacterial cultures was not complete, suggesting that some metabolites remained. Most metabolites were not predicted to contain S, since most of the S in the cultures was released as $SO_4^{=}$. Identification of metabolites from sulfolane proved to be difficult; these compounds appeared to be highly polar and likely consisted of 1-4 C. Soluble organic C remained in the cultures after sulfolane biodegradation was complete, however further research is required in order to identify the compounds that made up this detectable material.

Sulfolane-degrading isolates were tentatively identified (Table C1), however the identities appeared to be dependant on the tests used to identify each organism, suggesting that most standard identification procedures were not likely to have been accurate for these environmental isolates. All but one of the isolates were found to be Gram-negative rods, and the one Gram-positive isolate grew slowly on sulfolane. One sulfolane-degrading isolate was selected for extensive mineralization studies. This organism was identified using a combination of 16S rRNA sequencing and biochemical means, and was found to be most closely related *Variovorax paradoxus*. The match was poor, therefore WPW-S28 might be *V. paradoxus*, or it may represent an unidentified species in this genus. Only the species *paradoxus* has currently been described for the genus *Variovorax* (Holt et al., 1994; Kersters et al., 1991; Maidak et al., 1997).

4.3 Suggestions for future directions in research on sulfolane biodegradation

This thesis research was divided into two topics, both focusing on different aspects of sulfolane biodegradation. It will probably be most convenient to pursue further research in these two separate veins.

4.3.1 Future directions for site-oriented research

4.3.1.1 Microcosm studies

In this thesis research, most sulfolane biodegradation occurred under aerobic conditions. Supplementation with inorganic N and P sources increased the rate of biodegradation, and decreased lag times. Shake-flask cultures were used to predict the requirements for stimulation of microbial activity in 2.5-L and air-sparged microcosms. Previous research demonstrated that biological activity against xenobiotic compounds was similar in 2.5-L laboratory microcosms and *in situ* incubations. The next logical step in this research, therefore, would be a set of matching laboratory shake-flask cultures, 2.5-L microcosms, and *in situ* incubations. In order to decrease the amount of field work required, parallels between laboratory and field microcosms should be demonstrated; this would allow many predictions to be made in the laboratory and minimize the number of field situations to be tested.

The initial experiments should include replicate samples incubated in the laboratory and in the field. This thesis research demonstrated correlations between optimal nutrient requirements for sulfolane degradation observed in the shake-flask cultures and the 2.5-L or air-sparged microcosms inoculated with the same sediment samples. Shake-flask cultures should prove useful for predicting nutrient requirements at the contaminated site locations; these requirements could be further investigated using sediment microcosms incubated in the laboratory and *in situ*. The *in situ* microcosm design suggested by Christensen et al. (1995a) is well characterized and has been demonstrated to yield results similar to the 2.5-L laboratory microcosms (Christensen et al., 1995a; 1995b). As this thesis research has provided initial data for 2.5-L microcosms, this *in situ* design may be a good first step for field investigations.

4.3.1.2 Pilot scale remediation projects in the field

Once the capacity for sulfolane biodegradation in a contaminated aquifer has been understood, pilot scale field trials would offer a logical next step toward remediation of the site. Although sulfolane does not interact significantly with soils (Luther et al.,

1998) and therefore might be a good candidate for pump and treat remediation with a surface-level bioreactor, its co-contaminants from the Sulfinol® process, including DIPA, render this type of treatment of contaminated aquifers pointless. Sulfolane might be removed from the groundwater, yet some co-contaminant compounds would remain sorbed to aquifer sediments, slowly being released into the groundwater over time. An *in situ* remediation technique such as air sparging might prove to be more useful for overall site remediation. Blackburn and Hafker (1993) and Morgan and Watkinson (1989) describe a system for injecting nutrients and air into an aquifer upgradient from the contaminated zone. Once the nutrient requirements for sulfolane biodegradation were determined, it would be interesting to do a pilot scale pump and treat experiment on a sulfolane-contaminated region of an aquifer.

Due to its ability to migrate long distances in groundwater, sulfolane has a high potential for contamination off site. A system for groundwater treatment near the leading edge of the contaminant plume might control this problem. At this part of the plume, a system to remove groundwater from the surrounding aquifer for treatment might be feasible, as no compounds liable to remain bound to soil would be involved. Cocontaminants of sulfolane, such as DIPA, would not be likely to travel as far in the groundwater, due to interactions with soil (Luther et al., 1998) and their potential for anaerobic biodegradation (Gieg et al., 1998; Gieg et al., 1999). Treatment of the leading edge of the plume would be particularly effective in combination with air-sparging and nutrient supplementation in the more highly contaminated upgradient portions of the contaminated aquifer, to remove compounds such as DIPA. The most reasonable system for preventing off-site sulfolane contamination would be an inexpensive means for groundwater direction and containment, to remove sulfolane before groundwater migrated off site. An artificial wetlands system might be useful for this purpose; this thesis research demonstrated that inocula from wetlands were capable of degrading sulfolane. CAPP and Environment Canada have cosponsored an engineered wetlands pilot project at a gas plant site. The preliminary results from this project demonstrated good removal of BTEX compounds from wastewater (B. Moore, Komex International Ltd., Calgary, AB). A similar system could be used to remove sulfolane from contaminated groundwaters.

4.3.2 Future directions for pure culture and sulfolane metabolism research

4.3.2.1 Genetic studies of sulfolane-degrading organisms

One sulfolane-degrading bacterial isolate has currently been identified. Identification other isolates, preferably from different sites, would allow predictions to be made about the species that are able to utilize this compound. Identification of environmental isolates with standard kits such as the API 20E and API 20NE tests yielded poor results. Sequencing the 16S rRNA gene for each organism could provide more accurate placement of sulfolane-degrading organisms in the correct genus, if not species; these identities could be confirmed with a minimal number of biochemical tests.

Identification of one or more of the genes involved in sulfolane degradation could be important for describing the pathway for sulfolane catabolism, and determining whether most sulfolane-degrading species tend to use a similar pathway. Unique regions in a sulfolane-degrading gene sequence could be used as probes for the detection of sulfolane-degrading populations at contaminated sites, and would be particularly useful in for detecting viable but non-cultureable organisms.

4.3.2.2 Elucidation of a sulfolane degradation pathway

Experiments reported in this thesis have demonstrated that the detection of sulfolane metabolites in culture supernatants may be difficult. Some interesting approaches to the problem of trapping and identifying these metabolites are possible. Washed cell cultures are likely to be useful for metabolite work. Allison et al. (1995) successfully identified homovanillate metabolites from *V. paradoxus* washed cell cultures. Even the most vigorous sulfolane-degrading cultures tend to grow quite slowly; by harvesting cells during logarithmic phase growth and resuspending concentrated cells in a buffer solution, it should be possible to have a large percentage of the population in a similar state at the same time. Cell-free extracts (Allison et al., 1995; Cripps, 1973) may also prove useful for producing sulfolane metabolites. One challenge remains to be met: to produce each metabolite in sufficient quantities for detection and identification, and to identify these metabolites. Washed cell cultures or cell-free extracts should provide metabolites in quantity; it remains to develop a method for detecting the resulting compounds.

Compounds which are polar and acidic may become trapped on a basic ion exchange resin present in culture supernatants (J. Gallagher, University of North Dakota, personal communication). Derivatization would remove these compounds from the ion exchange resin; J. Gallagher suggested the use of iodomethane for this procedure. Metabolites present in the medium could also be collected by freeze drying culture supernatant, resuspending the sample in an appropriate (relatively polar) solvent and derivatizing. Given the results to date, acidification of culture supernatants before derivatization yielded better results than treatment with a base. Derivatization with a compound that provided a relatively large side chain would likely assist in detecting sulfolane metabolites, as they are likely to be of low molecular weight as well as polar. Solid phase extraction of metabolites from culture supernatants appeared not to work well; further investigation into this technique is required. Use of a different matrix for binding the metabolites might improve these results. If sulfolane metabolites could be detected by GC-FID or GC-FPD, analysis by GC-MS would allow identification of each compound.

A method for detecting thiols in water was reported by Vairavamurthy and Mopper (1990). These authors derivatized low molecular weight hydrophilic thiols: cysteine, glutathione, and 3-mercaptopropionic acid, using the derivatizing agent 2,2'-dithiobis(5-nitropyridine) (DTNP). The technique appeared to be extremely simple; DTNP reacts with thiols, sulfite and thiosulfate, adding a 5-nitropyridine group to the reactive S, and the reaction can be done directly in water. These authors used liquid chromatography to detect derivatized thiol compounds; this method may also prove useful for identifying and detecting sulfolane metabolites. The strong sulfurous odor produced by WPW-S28 during sulfolane suggested the production of a volatile, S-containing compound. This compound might be derivatized by DTMP, particularly in sealed cultures where the headspace gas could not escape.

The reported methods for trapping and detecting low molecular weight, polar metabolites of biodegradation are limited. The most important step for determining the pathway of aerobic sulfolane catabolism will be to detect and identify the metabolites of biodegradation of this compound. The first step in this identification process will be to develop a method for collecting and detecting these metabolites. The groundwork for this attempt was laid as a part of this thesis research; it remains to develop the tools to make this work useful.

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APPENDICES

Appendix A Detailed description of soils, aquifers, and contaminated sites

A.1 Contaminated aquifer sediments used in a project to screen for sulfolane biodegradation potential

Aquifer sediments were obtained from three sour gas processing plants (sites 1, 2 and 3). The characteristics of each site sample are summarized in Table 2.1.

A.1.1 Site 1 description

The gas plant located at site 1 is one of the largest gas plants in the province of Alberta. This plant was built in 1960 as a compressor facility and modified in 1961 to recover natural gas liquids. The Sulfinol® plant portion of this facility went into operation in 1966 (Fitzgerald and Richardson, 1966).

The plant is located in a varied topography; gently rolling plains are present to the north and east, while to the west a moderately steep slope leads to a river approximately 1.5 km away, and to the south a similar slope leads down to a reservoir about 1 km away.

Former landfill cells and unlined evaporation and surface runoff ponds led to groundwater contamination with sulfolane and DIPA downgradient from the site. In 1993, the ponds and landfill cells were excavated and sorted material was either shipped off site for disposal, or stored on site in a new landfill completed in 1996. Contaminant compounds such as chloride, sulfolane and dissolved organic C exist in the groundwater near the former landfills and water storage ponds.

A.1.2 Site 2 description

The site 2 gas plant is a Sulfinol® plant in southwestern Alberta, which started operation in the 1960s. Beneath the site are unconsolidated sands and clay-rich glacial tills, which vary in composition and thickness throughout the site. Weathered bedrock is present beneath the sand and clay aquifer components. The gas plant is in an elevated position on the site, therefore hydraulic gradients sufficient for downward movement of water-soluble contaminants exist, although some of the clay tills are relatively low permeability.

Samples were taken downgradient of the site 2 landfill (Figure 2.1), which was developed in the mid-1970's for disposal of processing wastes including Sulfinol® filters, spent catalyst and construction debris, and was capped in 1978. The hydrogeology of the site directs groundwater flow through and under the landfill. The groundwater eventually discharges into a wetland adjacent to a small creek about 100 m away from the landfills. The highest contaminant concentrations detected in groundwater outside of the landfill cells, in 1996, were 800 mg/L sulfolane and 300 mg/L DIPA (immediately downgradient of the landfill cells). At those sites, the dissolved O_2 was below the detection limit. In groundwater samples taken upgradient of the landfill, where no sulfolane or DIPA contamination has been detected, the groundwater contained dissolved O_2 at 6 to 8 mg/L.

Two aquifer sediment core samples were collected from site 2. One core was obtained downgradient from a landfill that is a source of sulfolane and DIPA contamination in the groundwater; and was sulfolane and DIPA contaminated (Table 2.1). A second core sample was obtained upgradient from the landfill cells; this sample was not contaminated with sulfolane or DIPA (Table 2.1). The contaminated core sample was taken northeast from the east plume sampling site shown in Figure 2.1, in the zone with the same level of sulfolane contamination as the marked sampling site. The uncontaminated sample was taken from the same place as the uncontaminated sample shown in Figure 2.1. Core samples were cut into 1.5 m segments and shipped to the laboratory in 6-cm diameter plastic tubes. The core material used for the experiment was from the 3 to 4 m depth. Samples were stored at 4°C until used to inoculate microcosms. Groundwater samples collected from piezometers located near the core sample sites were used to determine the sulfolane concentration.

A.1.3 Site 3 description

The gas plant located at site 3 is in central British Columbia, on a plateau 460 m above the floor of a valley. This plant was commissioned in 1980 and expanded in 1994 to process sour natural gas, using the Sulfinol® process.

The plant site slopes gently from northeast to southwest, with groundwater drainage proceeding toward a creek. The soil and sediment materials at the site consist of morainal blanket or morainal veneer deposits, with a shallow groundwater system controlled by the bedrock surface geometry The bedrock at the site is highly fractured and weathered. The offsite drainage route initially existing at the site appears to have been altered from its original course by fill placed during construction of the plant.

A sulfolane- and DIPA-contaminated sample was taken downgradient from the filter and catalyst dump in the southwest corner of site 3. The samples contained bedrock consisting of sandstone, siltstone and shale (Luther et al., 1998).

A.2 Uncontaminated soils used to screen for sulfolane biodegradation potential

Three uncontaminated soils were used in the biodegradation potential screening experiments. One was taken from site 2, and is described in Table 2.1. An uncontaminated, humus-rich soil was taken from the Dh horizon of a region 6 km north of St. Albert, AB (Luther et al., 1998). This soil was sifted and air dried, then stored at room temperature until cultures were inoculated. A third sample of uncontaminated soil was obtained from a greenhouse at the University of Alberta (Edmonton, AB).

A.3 Aquifer sediments, wetland materials, groundwaters and surface waters used to determine the nutrient requirements for sulfolane degradation

Samples for the microbial population studies and nutrient requirement screening experiments were taken from five regions of site 2. The sampling locations included: aquifer sediments and groundwaters from two sulfolane and DIPA contaminated plumes downgradient from two different landfill cells, wetlands materials and surface waters taken from each of the two wetlands sites downgradient from the two contaminant plumes, and an uncontaminated aquifer sediment and groundwater sample taken upgradient from the landfill cells. The sampling locations are shown in Figure 2.1; the characteristics of each are summarized in Table 2.2. The C, N and P content of the aquifer sediments are shown in Table 2.3.

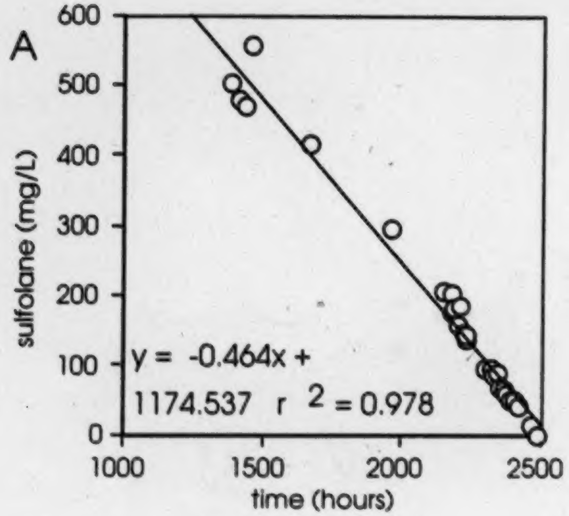
A.4 Aquifer sediments and topsoil used for air-sparged microcosm experiments

Sulfolane-contaminated sediment samples composed of sandy clay till were taken next to two Sulfinol® plant buildings on site 2. The Sulfinol® #1 sediment consisted of 35 to 60% coarse material. The Sulfinol® #2 sediment was 5% coarse fill material, and had a very strong odor (B. Samis, Komex International, Calgary, AB). Both sediment samples are described in Table 2.4, their C, N and P content is listed in Table 2.3. The Sulfinol® #2 soil was used for air-sparged microcosm experiments.

Sulfinol® soil samples were not homogeneous; they contained a number of rocks ≥ 1-cm in diameter, large pieces of hardened mud, and clumps of material that appeared to be saturated with a heavy hydrocarbon-containing substance, such as oil or tar. Before use samples were transferred to a large, sterile Pyrex dish. Rocks with a diameter ≥ 0.5 cm were removed, and the remaining sample was homogenized, then stored at 4°C. The pristine soil sample was a commercially available product from Olds College (Olds, AB). It contained large pieces of plant material; which were removed and discarded. For both contaminated and pristine soils, no particles larger than 0.5 cm in diameter were used in the landfarming chamber or shake-flask experiments.

Appendix B Details of the kinetic analyses of sulfolane degradation

In order to compare the sulfolane biodegradation in different experimental series, and the rates of biodegradation in different types of cultures and microcosms, the biodegradation rates for sulfolane were determined. The method of Metcalf and Eddy (1979) was used. Complex rate analyses were not attempted. Instead, the decrease of sulfolane over time in each culture or microcosm (excluding the lag time) was used in zero-order and first-order rate plots. A sample pair of rate plots is shown in Figure A1. The R^2 (a measure of the exactness of fit of the data points to the best fit line) for each set of plots was compared to determine which kinetic order best described sulfolane biodegradation in a given culture or microcosm. If the best fit was obtained with the plot of the substrate concentration decrease over time, the culture was considered to have degraded sulfolane following zero-order kinetics. If the best fit was obtained with the plot of the natural logarithm of substrate concentration decrease over time, the culture was considered to have degraded sulfolane following first-order kinetics. This information is summarized in Tables A1 to A5. More complex kinetic analyses would have been required to determine the exact kinetic model to describe sulfolane degradation in each case. Since few cultures or microcosms were duplicated, small variations in the data may have affected biodegradation such that a more complex kinetic model may not have been accurate. For the purposes of comparison, zero-order or first-order kinetics were felt to be sufficient. Further research would be required to generate sufficient data for complex kinetic analyses. Kinetic values for all the soil- or sediment-inoculated experiments were determined. No kinetic analyses of pure culture growth were done. In each table, the best value (zero-order or first-order) for each culture or microcosm is shown in bold face. For the analyses reported here, in almost all cases sulfolane biodegradation followed zero-order kinetics.



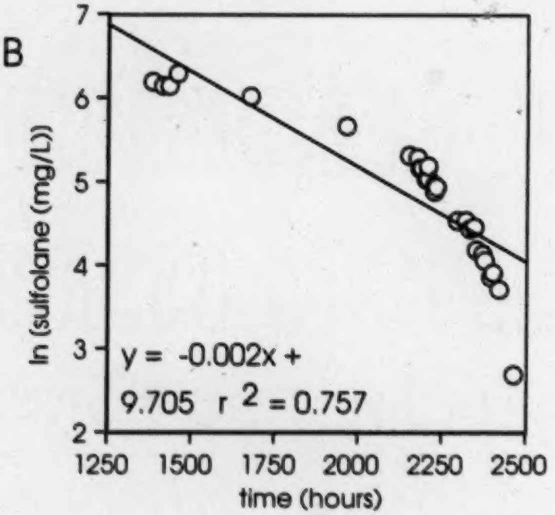


Figure B1. Sample of graphs used to determine the kinetic order of sulfolane biodegradation and the rate of removal of the compound. East contaminated plume, no N or P supplementation. A. Zero-order plot. B. First-order plot. The fit (r^2) was better for the zero-order plot.

Table B1. Summary of zero-order and first-order kinetic analyses of sulfolane biodegradation in shake-flask cultures inoculated with aquifer sediment or wetland materials from five sampling points at site 2. The more appropriate rate constraint for sulfolane degradation (determined by the R^2 value for the line) is shown in bold type.

		zero order		first order	
sample	N and P added	rate (mg/L/h)	R^2	rate (h^{-1})	R^2
east plume	Y[a]	**6.0**	0.885	0.016	0.816
	Y[b]	**3.7**	0.919	0.021	0.738
	N	**0.6**	0.945	0.003	0.658
west plume	Y[a]	**10.3**	0.965	0.028	0.918
	Y[b]	**5.2**	0.886	0.024	0.586
	N	0.4	0.878	**0.001**	0.915
uncontaminated	Y[a]	**1.4**	0.920	0.028	0.848
	Y[b]	**1.7**	0.920	0.024	0.811
	N	**0.4**	0.957	0.009	0.814
west wetland	Y[a]	**1.1**	0.924	0.011	0.897
	Y[b]	**0.3**	0.952	0.005	0.886
	N	**0.2**	0.970	0.007	0.883
east wetland	Y[a]	**1.1**	0.915	0.018	0.894
	Y[b]	**0.3**	0.833	0.009	0.626
	N	**0.2**	0.942	0.007	0.915

Table B2. Summary of zero-order and first-order kinetic analyses of sulfolane biodegradation in shake-flask cultures inoculated with aquifer sediment and groundwater from the east and west plume contaminated regions at site 2 and supplemented with various amounts of N or P. The appellations N_r and P_r represent the concentrations of N and P required to achieve a C:N:P ratio of 100:5:1. The more appropriate rate constraint for sulfolane degradation (determined by the R^2 value for the line) is shown in bold type.

	nutrients added		zero order rates		first order rates	
sample	P_r	N_r	(mg/L/h)	R^2	h^{-1}	R^2
east plume	0	0	**0.5**	0.978	0.002	0.757
		0.8	**0.4**	0.958	0.002	0.775
		1.6	**0.6**	0.970	0.004	0.831
	0.8	0	**1.5**	0.947	0.006	0.824
		0.8	**2.1**	0.917	0.012	0.728
		1.6	**2.0**	0.981	0.011	0.847
	1.6	0	**3.0**	0.972	0.020	0.705
		0.8	**1.8**	0.959	0.007	0.764
		1.6	**2.9**	0.963	0.014	0.855
	2.4	1.6	**3.0**	0.919	0.015	0.769
	3.2	1.6	**1.6**	0.941	0.024	0.661
	4.0	1.6	**4.8**	0.933	0.023	0.839
	705	1.6	**4.5**	0.903	0.019	0.775
west plume	0	0	**1.4**	0.896	0.006	0.723
		0.8	**1.0**	0.982	0.004	0.776
		1.6	**1.0**	0.987	0.004	0.861
	1	0	**4.4**	0.989	0.018	0.806
		0.8	**3.9**	0.976	0.014	0.790
		1.6	**3.8**	0.991	0.017	0.828
	2	0	**6.3**	0.954	0.028	0.784
		0.8	**10**	0.974	0.036	0.864
		1.6	**12**	0.997	0.070	0.793
	750	1.6	**6.2**	0.976	0.016	0.935

Table B3. Summary of zero-order and first-order kinetic analyses of sulfolane biodegradation in 2.5-L microcosms consisting of aquifer sediments and the corresponding groundwater or wetland materials and the corresponding surface water. The appellation P_r represent the concentration of P required to achieve a C:N:P ratio of 100:5:1. The more appropriate rate constraint for sulfolane degradation (determined by the R^2 value for the line) is shown in bold type.

	zero order rates		first order rates	
microcosm	(mg/L/h)	R^2	h^{-1}	R^2
east plume + P[a]	**0.5**	0.949	0.003	0.665
west plume + P[a]	**0.3**	0.985	0.002	0.822
west wetland	**0.3**	0.988	0.008	0.884
east wetland	**0.4**	0.921	0.008	0.764

[a] 1P_r was added to the microcosm after 221 d of incubation without any added nutrients.

Table B4. Summary of zero-order and first-order kinetic analyses of sulfolane biodegradation in shake-flask cultures inoculated with aquifer sediment from near Sulfinol® building #2 or with topsoil. Low N or P refers to sufficient N or P to account for the sulfolane-C in a sample, according to the C:N:P ratio of 100:5:1. High N or P refers to sufficient N or P to account for the total C in a sample. The more appropriate rate constraint for sulfolane degradation (determined by the R^2 value for the line) is shown in bold type.

sample	nutrient amendment	zero order rate (mg/L/h)	zero order R^2	first order rate (h^{-1})	first order R^2
Sulfinol® sediment	no NP	**0.2**	0.985	0.003	0.858
	low N	**0.6**	0.881	0.012	0.746
	low P	**0.1**	0.852	0.002	0.608
	low NP	**0.4**	0.966	0.007	0.872
	high N	**0.6**	0.984	0.012	0.941
	high P	ND[a]	ND[a]	ND[a]	ND[a]
	high NP	**2.0**	0.951	NA[b]	NA[b]
	B+N8P	**1.2**	0.967	0.025	0.776
	trace	**0.1**	0.974	0.002	0.855
topsoil	no NP	**1.5**	0.971	NA[b]	NA[b]
	low N	**1.5**	0.971	NA[b]	NA[b]
	low P	**1.6**	0.968	NA[b]	NA[b]
	low NP	**1.8**	1.000	NA[b]	NA[b]
	high N	**0.1**	0.800	0.002	0.577
	high P	**2.3**	1.000	NA[b]	NA[b]
	high NP	**2.1**	1.000	NA[b]	NA[b]

[a] ND no sulfolane degradation was detected in this culture

[b] NA not applicable. Sulfolane degradation in this culture was completed before adequate time points for first order kinetic analysis were collected.

Table B5. Summary of zero-order and first-order kinetic analyses of sulfolane biodegradation in landfarming chamber microcosms inoculated with aquifer sediment from near Sulfinol® building #2 or with topsoil. The more appropriate rate constraint for sulfolane degradation (determined by the R^2 value for the line) is shown in bold type. In cases where very little difference between zero-order and first-order kinetics was observed, both values are shown in bold type.

sample	nutrient amendment	zero order rate (mg/L/h)	zero order R^2	first order rate (h^{-1})	first order R^2
Sulfinol® sediment	no N or P	**0.366**	0.966	**0.002**	0.960
	N	**0.323**	0.979	0.001	0.918
	P	0.188	0.909	**0.001**	0.940
	N and P	**0.620**	0.964	**0.004**	0.966
topsoil	no N or P	**0.561**	0.931	0.009	0.717
	N	**0.310**	0.836	0.008	0.607
	P	**0.345**	0.926	0.008	0.806
	N and P	**0.850**	0.914	0.017	0.783

Appendix C Details of sulfolane degradation by bacterial cultures

C.1 Enrichment of sulfolane-degrading bacteria in shake-flask cultures

A total of five aquifer sediment or wetland samples were used to document the enrichment of sulfolane-degrading bacteria in sediment-inoculated shake-flask cultures. The cultures initially consisted of 50 g (wet weight) of wetland materials or aquifer sediments, and 200 mL of the corresponding groundwater or surface water. For cultures consisting of uncontaminated groundwaters, sulfolane and DIPA were added as substrates. The sulfolane concentration in the cultures was monitored by GC. Each culture was given a second dose of sulfolane, once the first had been utilized. No further DIPA was added to the cultures at this time. After the second dose of sulfolane was gone, transfer cultures consisting of 10 mL sediment culture and 200 mL B+N8P medium were made. The population of sulfolane-degrading bacteria and total cultureable heterotrophs (bacteria and fungi) were monitored using PAT medium (Section 3.4). This experiment was reported in Section 3.4.3.3; the figure for the east plume transfer culture was shown (Figure 3.14). The graphs shown in Figure B1 demonstrate the data for all the cultures and transfer cultures that were monitored.

Initially, the sulfolane-degrading bacterial populations were relatively small in the cultures inoculated with contaminated aquifer sediment, when compared with the total heterotrophic populations (Figures B1A and C). No sulfolane-degrading population was initially detected in the uncontaminated aquifer sediment or wetland cultures (Figures B1E, G and I). The sulfolane-degrading populations found in all the transfer cultures represented a much larger proportion of the total heterotrophic bacterial population than in the original cultures (Figure B1). In all cases but the east wetland culture, the sulfolane-degrading population represented almost all the total cultureable heterotrophs. This was particularly evident in the uncontaminated aquifer sediment culture, where few organisms other than sulfolane-degraders were observed in the transfer culture. The sulfolane-degrading bacteria in this culture all produced the same colony type, therefore after a single transfer this culture was almost pure; typical mixed cultures demonstrated at least 10 colony types on PAT medium. Overall, these data showed that sulfolane-degrading bacterial populations were present in contaminated aquifer sediments, and that in uncontaminated soils or sediments, a sulfolane-degrading population was stimulated to develop, which agreed with the findings in other sediment-inoculated cultures and microcosms. This experiment also demonstrated that the enrichment of

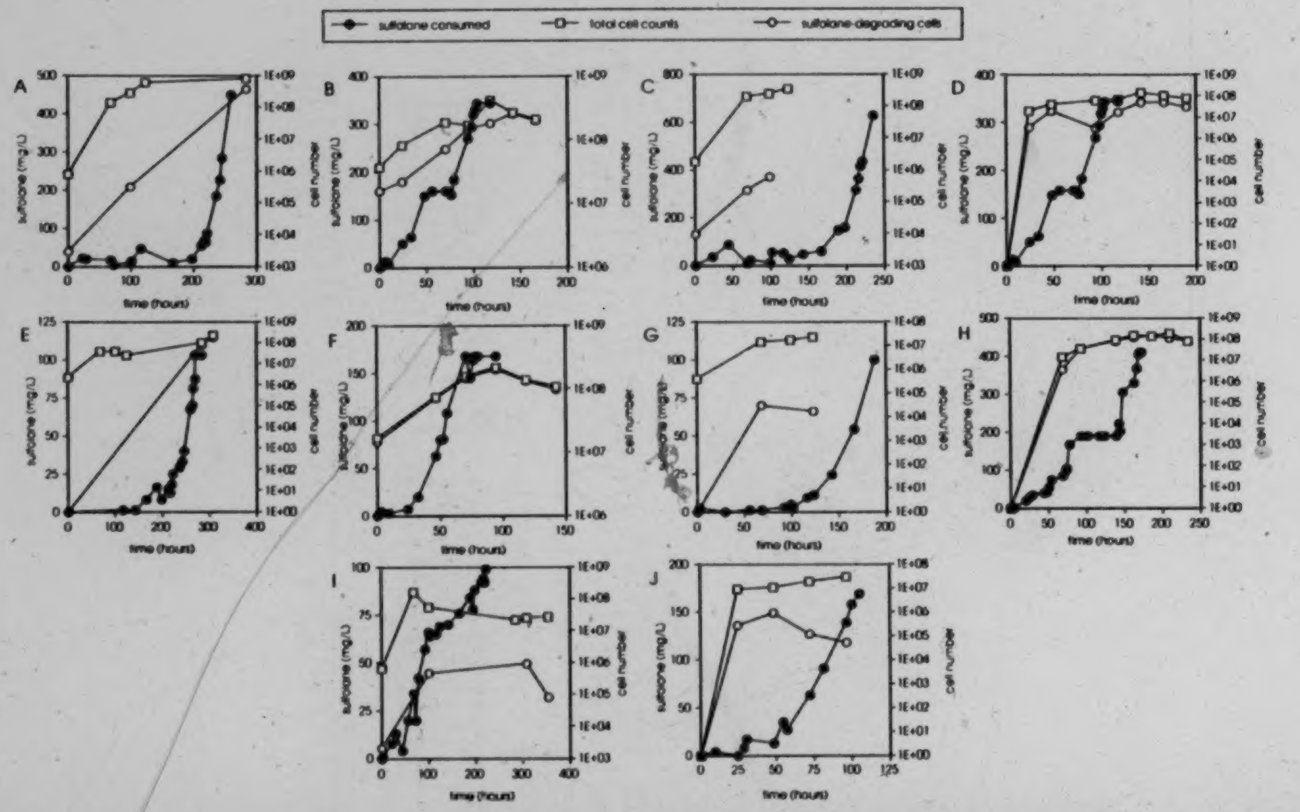


Figure C1. Changes in the number of total cultureable heterotrophs and sulfolane-degrading bacteria during the biodegradation of sulfolane in cultures consisting of site 2 materials and groundwaters, or transfer cultures consisting of 10 mL original culture and B+N8P, incubated at 10°C. A. East plume. B. East plume transfer. C. West plume. D. West plume transfer. E. Uncontaminated. F. Uncontaminated transfer. G. West wetland. H. West wetland transfer. I. East wetland. J. East wetland transfer.

sulfolane-degrading bacteria could be tracked in a population of heterotrophic bacteria, using PAT medium.

C.2 Pure sulfolane-degrading bacterial cultures, their growth on sulfolane, and the corresponding decrease in culture pH

A total of ten pure, sulfolane-degrading bacterial cultures were isolated and maintained for characterization. Six of these cultures were isolated from site 1; their growth on sulfolane and the corresponding decrease in pH of the culture medium is shown in Figure B2. Figure B3 shows the growth of pure bacterial isolates from site 2 on sulfolane. The cell and colony morphologies of each isolate, and tentative identities for some, are summarized in Table B1. All but one of the bacterial isolates were Gram-negative bacilli; the other isolate was found to be Gram-positive cocci. Most of the bacterial isolates initially degraded sulfolane vigorously, however approximately half these isolates lost this level of activity after a number of serial transfers. The bacterial isolates were maintained in minimal medium; it is possible that some were auxotrophic for certain substances, or that the cultures were not really pure and a second, undetectable organism that was essential for their growth was slowly lost. The possibility that some organisms were auxotrophic is not likely, since it often took a year or more for the degradative abilities of these isolates to wane; no component of the original cultures could have lasted at useable concentrations after isolation on agar plates, followed by a number of serial transfers. However, many of the incubation conditions in the laboratory may have been detrimental to the growth of these organisms.

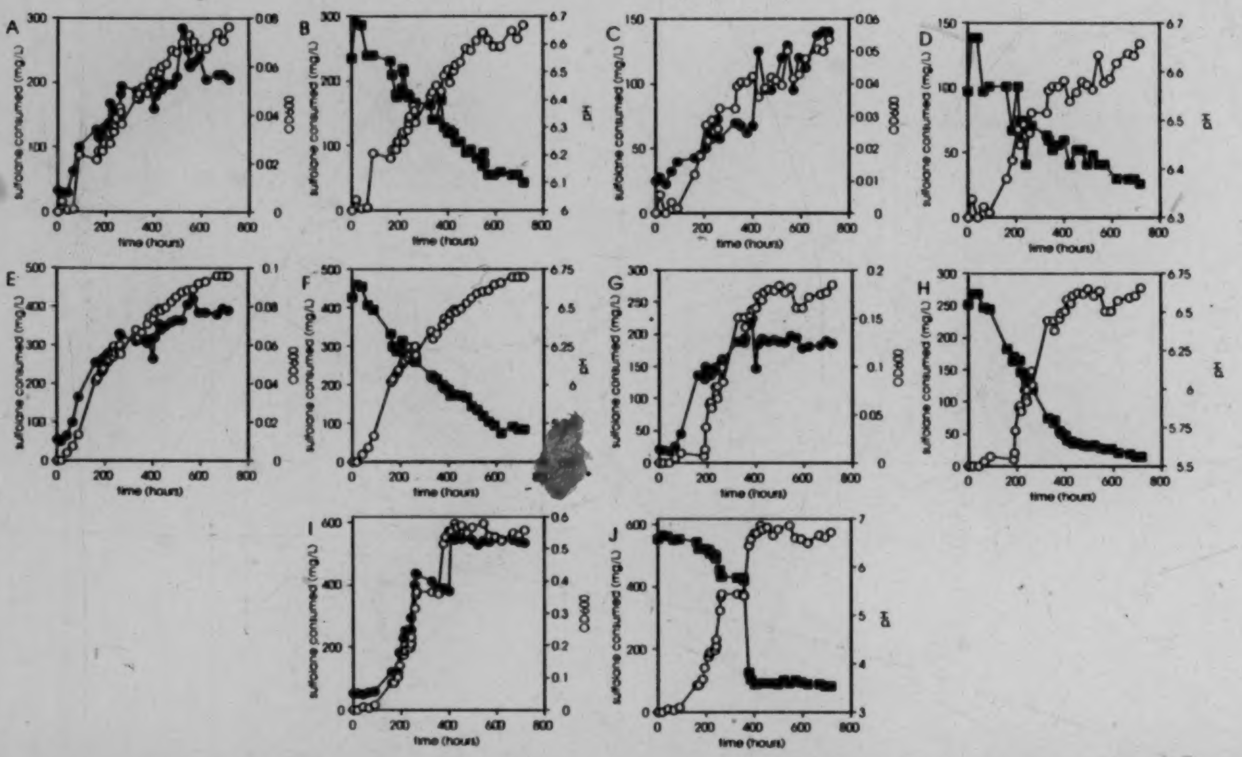


Figure C2. Sulfolane utilization by pure bacterial cultures grown in B+N8P medium and the corresponding increase in OD_{600} (closed circles) and decrease in pH (closed squares). Open circles show the sulfolane consumed. All cultures were incubated at 28°C except HP5-S8, which was incubated at 8°C. A. HP1-S28: sulfolane and OD_{600}. B. HP1-S28: sulfolane and pH. C. HP8-S28: sulfolane and OD_{600}. D. HP8-S28: sulfolane and pH. E. HP81-S28: sulfolane and OD_{600}. F. HP81-S28: sulfolane and pH. G. WP1-S28: sulfolane and OD_{600}. H. WP1-S28: sulfolane and pH. I. HP5-S8: sulfolane and OD_{600}. J. HP5-S8: sulfolane and pH.

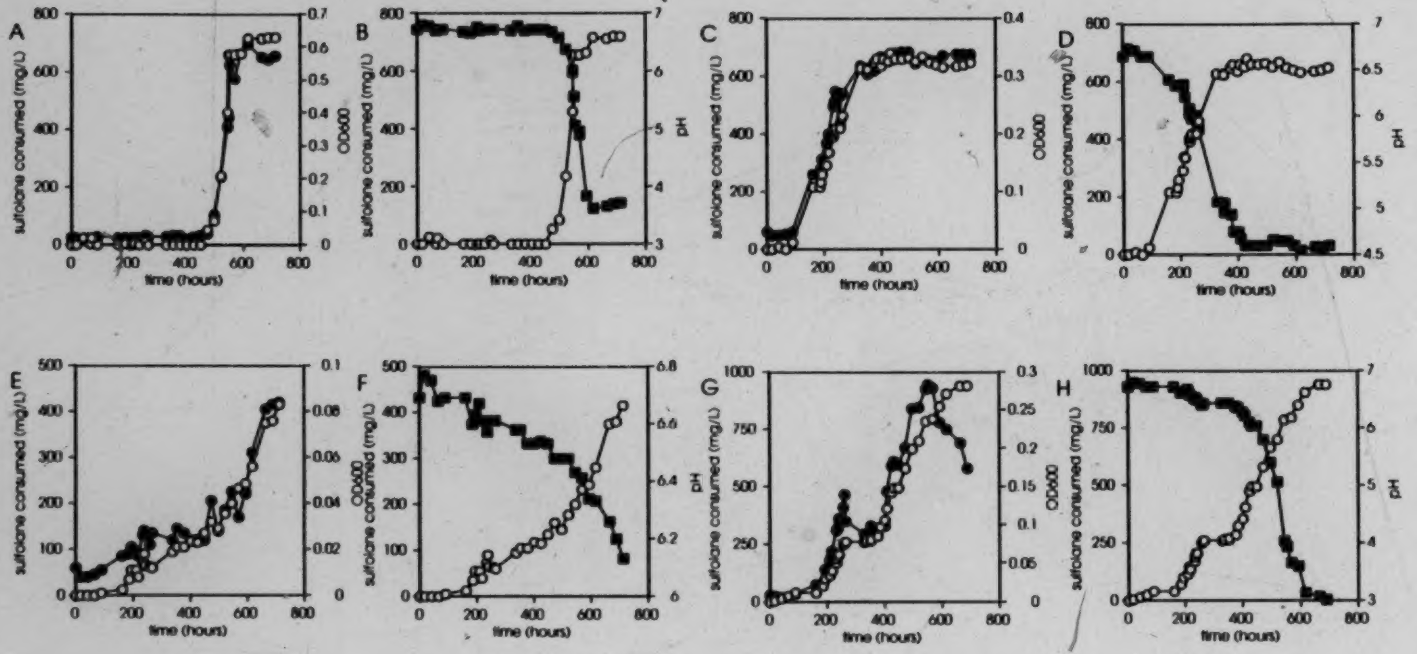


Figure C3. Growth of sulfolane-degrading isolates from site 2 on sulfolane at 28°C in OD_{600} (closed circles), and the corresponding change in pH of the B+N8P medium (closed squares). Open circles show the total sulfolane consumed. A. WPW-S28: sulfolane and OD_{600}. B. WPW-S28: sulfolane and pH. C. WPU-S28: sulfolane and OD_{600}. D. WPU-S28: sulfolane and pH. E. WPWS-S28: sulfolane and OD_{600}. F. WPWS-S28: sulfolane and pH. G. WPES-S28: sulfolane and OD_{600}. H. WPES-S28: sulfolane and pH.

Table C1. Descriptions of sulfolane-degrading isolates. Cells were grown on LB agar to determine the colony morphology. Samples of each colony were Gram stained. The identities of organisms were determined by the Microbiology Research and Development Laboratory (Alberta Research Council, Vegreville, AB), B. Steen (Department of Microbiology and Immunology, University of British Columbia, Vancouver, BC), by use of the API E and API NE bacterial identification kits; or by 16S rRNA sequencing and supplementary biochemical tests.

culture designation	Gram stain	colony morphology	possible isolate identities
HP1-S28	negative rods, 1:3, rounded ends, no visible grouping	circular, 1-2 mm, umbonate, beige, shiny opaque	*Methylobacterium mesophilicum*[a], *Burkholderia cepacia*[b]
HP5-S8	negative rods, 1:3, rounded ends, singles, end to end pairs	spindled, irregular, 2-3 mm, raised, pale yellow, opaque, translucent edges	*Comomonas acidovorans*[a], *Variovorax (Alcaligenes) paradoxus*[c]
HP8-S28	positive cocci, singles and pairs	circular, 1-2 mm, raised, creamy beige, sticky, opaque	no identification was attempted
HPRK2-S28	negative rods, 1:3, no visible grouping	circular, 3-4 mm, umbonate, beige, dull, opaque	no identification was attempted
WP1-S28	negative rods, 1:3, rounded ends, no visible grouping	circular, 2-3 mm, umbonate, beige, dull, opaque, sticky	*Pseudomonas* spp.[a], *Burkholderia cepacia*[b]
WPES-S28	negative rods, 1:3, no visible grouping	irregular, 3-5 mm, flat, creamy, shiny, opaque, sticky	*Pseudomonas putida*[d], *Acinetobacter* spp.[d], *Pseudomonas aeruginosa*[e], *Burkholderia cepacia*[e]
WPU-S28	negative rods, 1:1.5 or 1:2, end to end pairs and chains	circular, 2 mm, flat, beige, dull, opaque	*Pseudomonas mesophilica*[c], *Pseudomonas fluorescens*[e]

Table C1 continued.

culture identity	Gram stain	colony morphology	possible isolate identities
WPW-S28	negative rods, 1:3, rounded ends, no visible grouping, no flagella	spindled, 3-5 mm, umbonate, pale yellow, shiny, sticky opaque	*Listonella damsela*[d], *Pasteurella pneumotropica*[d], *Pseudomonas fluorescens*[e], *Variovorax (Alcaligenes) paradoxus*
WPWS-S28	negative rods, 1:3 or 1:4, singles, end to end pairs	oval, 2-3 mm, raised, beige, sticky, opaque	*Comomonas acidovorans*[d], *Acinetobacter calcoaceticus*[e]

[a] The identity of this organism was determined by the Microbiology Research and Development Laboratory (Alberta Research Council, Vegreville, AB).

[b] More complete testing by the Microbiology Research and Development Laboratory (Alberta Research Council, Vegreville, AB) determined that this organism was *Burkholderia cepacia*, however B. Steen (Department of Microbiology and Immunology, University of British Columbia, Vancouver Children's Hospital, Vancouver, BC) determined that this organism was not *B. cepacia*; *B. cepacia* has a slow, weak oxidase positive reaction while HP1-S28 was oxidase negative and WP1-S28 demonstrated a fast, strong oxidase positive reaction.

[c] More complete testing by the Microbiology Research and Development Laboratory (Alberta Research Council, Vegreville, AB) determined that this organism was *Variovorax (Alcaligenes) paradoxus*.

[d] Organism was identified using the API NE kit.

[e] Organism was identified using the API E kit.

[f] Organism was identified using a combination of 16S rRNA sequence results, and biochemical and morphological tests.

Appendix D Attempts to identify metabolites from sulfolane degradation

Partial mineralization of sulfolane to CO_2 and $SO_4^=$ and conversion to biomass under aerobic conditions by mixed and pure bacterial cultures was demonstrated (Sections 3.8.1-3.8.3), however complete mineralization of the compound was not observed for any of the pure cultures. Thus, some metabolites of sulfolane biodegradation, other than CO_2 and $SO_4^=$, must exist. Pure cultures mineralized less sulfolane than mixed cultures, suggesting that some of the potential metabolites might be utilized by different bacterial species than those that were initially able to degrade sulfolane. Hence, pure cultures were likely to be more useful for the elucidation of a pathway for aerobic sulfolane degradation; the incomplete mineralization of the compound by these cultures suggested that one or more metabolites might accumulate in the culture medium over time. With this view in mind, an effort was made to detect and identify potential sulfolane metabolites produced by a pure, sulfolane-degrading culture. The culture WPW-S28 was selected for metabolite studies; this culture did not completely mineralize sulfolane and yet it grew vigorously using the compound as a sole source of C and S. Growth curves (OD_{600} vs time) were reasonably reproducible for WPW-S28, provided that the culture was transferred biweekly. Many of the pure, sulfolane-degrading cultures did not grow as well as WPW-S28, thus had less reproducible results for growth, which would have been inconvenient for any time course experiments involving metabolite production.

Two different approaches toward identification of a sulfolane biodegradation pathway were taken. The first was an attempt to disrupt WPW-S28 genes involved in sulfolane degradation. The second was to detect sulfolane metabolites in the culture supernatants of WPW-S28.

D.1 Attempts to disrupt sulfolane-degrading genes in the pure isolate WPW-S28

The disruption of a gene involved in sulfolane degradation by WPW-S28 could have two useful results. One would be the buildup of one or more intermediate transient metabolites in sulfolane degradation, assuming that the first step in the degradation pathway, at least, was not affected. The second useful consequence of gene disruption would be that the disrupted gene could be sequenced, using the source of gene disruption as a basis for developing sequencing primers. This gene could also be cloned for further study (Singh and Klingmüller, 1986). If the gene sequence was available, one could predict the possible activity of the gene product by comparing this sequence with databases containing known gene sequences, using sequence alignment tools such as

BLAST. Familiarity with the enzymes involved in sulfolane degradation would allow predictions of potential metabolites.

The first step toward gene disruption was to demonstrate that no plasmids were present in WPW-S28. Many biodegradation genes are plasmid-encoded; as most plasmids exist in multiple copies in a cell it would be difficult to disrupt all the copies of any gene in such a system, therefore a plasmid-containing bacterial species would not be ideal for this experiment. Plasmids also can be involved in complex exclusion systems, which might prevent the introduction of another plasmid into these cells. The method employed for gene disruption in WPW-S28 involved a plasmid; this method could therefore be complicated by the presence of native plasmids in WPW-S28.

Plasmid DNA extractions were done on WPW-S28; no plasmid was found in this organism. A culture of *E. coli* known to contain a plasmid was extracted simultaneously as a positive control. Plasmid preparations were duplicated twice by P. Beatty (Dept. of Biological Sciences, University of Alberta, Edmonton, AB), using cultures of WPW-S28 grown on sulfolane as a sole source of C and S, in order to ensure that these results were reproducible.

D.1.1 Description of Escherichia coli HB101 pGS9

A culture of *E. coli* strain HB101 with a plasmid carrying a transposon for host mutagenesis was obtained from J. Foght (Dept. of Biological Sciences, University of Alberta, Edmonton, AB). This organism possessed pGS9 (Figure D1), a plasmid that was designed by Selvaraj and Iyer (1983). The plasmid pGS9 was constructed from the *E. coli* plasmid p15A, a cryptic and nonconjugative plasmid. It has a narrow host range origin of replication, therefore is unlikely to be maintained in organisms other than *E. coli*. A gene for chloramphenicol resistance, a transfer region from the N plasmid group pCU1, which allows conjugation between species, and a copy of the transposon Tn5 are all present on this plasmid (Figure D1). The transposon Tn5 carries the genes for kanamycin, bleomycin and streptomycin resistance (Bennet, 1991). The use of Tn5 for random mutagenesis is advantageous as it inserts into relatively random DNA sequences; some transposons are more selective about the sequences into which they integrate (Bennet, 1991). Due to the narrow host range of pGS9, most recipient organisms will not be able to maintain this plasmid (Selvaraj and Iyer, 1983). However, Tn5 may be able to integrate into the host chromosome before the plasmid is lost; in these cases the recipient organism will demonstrate resistance to kanamycin, bleomycin and streptomycin. The plasmid-bearing *E. coli* will demonstrate resistance to

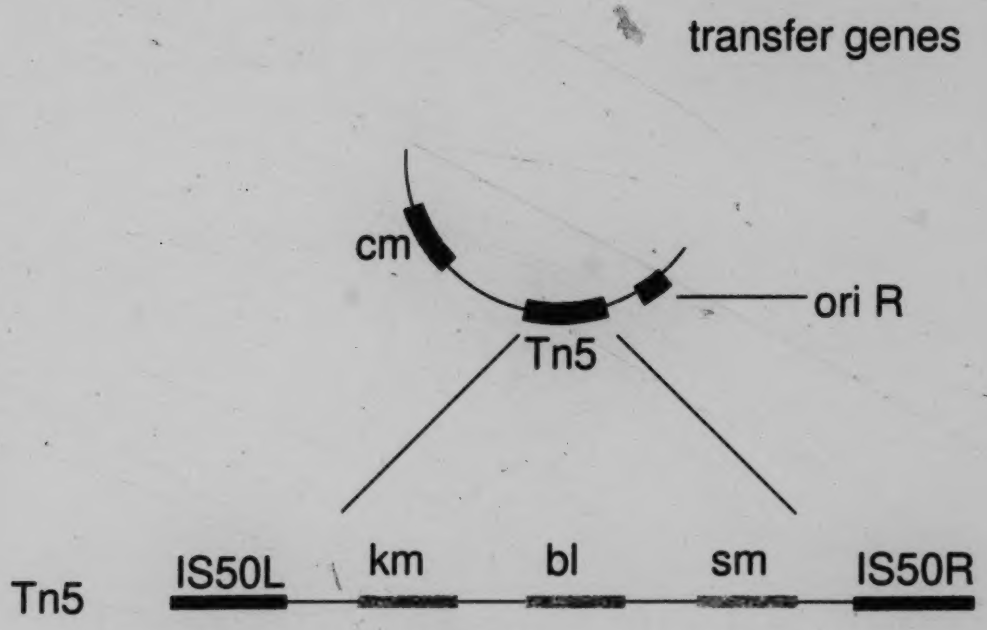


Figure D1. The pGS9 suicide vector used for transposon mutagenesis (taken from Selvaraj and Iyer, 1983). The plasmid carries a narrow host range origin of replication which functions in *E. coli*, and a copy of the transposon Tn5. The plasmid genes are indicated as follows: N transfer genes allow transfer of the plasmid to different hosts; ori R describes the origin of plasmid replication; cm is the gene for chloramphenicol resistance; and transposon 5 is represented by Tn5. The structure of Tn5 is shown below the plasmid (taken from Bennet, 1991). Tn5 has genes for kanamycin, bleomycin and streptomycin resistance (km, bl and sm, respectively) between two 1.5 kb insertion sequences (IS50L and IS50R). The right insertion sequence, IS50R, encodes the transposase genes (responsible for integration of Tn5 into host DNA); the left insertion sequence (IS50L) contains the promoters responsible for transcription of the antibiotic resistance genes.

chloramphenicol, and the antibiotic resistance conferred by Tn5. Kanamycin resistance can be used to select recipient organisms, in conjunction with a selective method that will not allow *E. coli* to grow. The methods for this project are in Section 2.13.1.

D.1.2 Development of a selection method for WPW-S28 transformants

The strain of *E. coli* HB101 carrying pGS9 was *pro*$^{-}$ *leu*$^{-}$ *thy*$^{-}$ *thi*$^{-}$. Due to these auxotrophic traits, this bacterium should be unable to grow on minimal medium with a simple sugar as a sole C source. WPW-S28 was demonstrated to grow on agar plates consisting of B+N8P with glucose as the sole C source. As predicted, *E. coli* HB101 pGS9 was not able to grow on this medium. With this information, a selective medium for WPW-S28 with Tn5 from pGS9 integrated into the chromosome was designed. This medium consisted of 15 g agar, 4 g glucose, 1 g sulfolane and 50 mg kanamycin (kan) per 1 L B+N8P medium, and was called GK medium. Sulfolane was added to the medium in order to maintain the ability of WPW-S28 to degrade this compound unless any genes involved in its degradation had been disrupted; in the absence of selective pressure to maintain this ability it might be lost. Sulfolane did not support the growth of *E. coli* HB101 pGS9. WPW-S28 was demonstrated to be sensitive to kan. The purpose of the transposon mutagenesis experiment was to disrupt sulfolane-degrading genes; it would be difficult to differentiate between WPW-S28 that had lost the ability to degrade sulfolane naturally and WPW-S28 in which sulfolane degradative genes had been disrupted by Tn5.

D.1.3 Mating experiments with E. coli HB101 pGS9 and WPW-S28

Three types of mating between WPW-S28 and *E. coli* HB101 pGS9 were tested: cross-streak mating, Eppendorf mating and filter mating. Cross-streak mating was the simplest; LB agar plates were streaked with parallel lines of *E. coli* HB101 culture in one direction and parallel lines of WPW-S28 perpendicular to the *E. coli* lines (Owen and Ward, 1985). Cells were harvested from the regions where the two bacterial cultures crossed. Eppendorf mating was carried out using a modified version of the method described by Singh and Klingmüller (1986). The method was modified to accommodate relatively poor growth by WPW-S28 when compared to growth of *E. coli*. The third type of mating was the filter mating method described by Selvaraj and Iyer (1983). This method was also modified to account for the relatively low levels of cell growth in WPW-S28 cultures when compared to cultures of *E. coli*. Trial runs suggested that filter mating yielded the greatest number of cells on GK agar, therefore the other two techniques were no longer used.

The transconjugation frequency was calculated based on the total number of WPW-S28 CFU/mL in the transformation mix. Typical WPW-S28 counts for the filter mated cell resuspension were 2 x 10^9 CFU/mL. Approximately 25 to 50 colonies were observed on GK agar plated with 0.1 mL of this mixture, therefore the frequency at which WPW-S28 obtained kan^R was between 1.25 x 10^{-7} and 2.5 x 10^{-7}. Two events were thought to be required for kan^R to be conferred on WPW-S28; first, the cells had to receive pGS9 from *E. coli* HB101 pGS9, then Tn5 from the plasmid had to integrate into the chromosome of the transformed cells. The transconjugant frequency for *Azospirillum brasilense* was 1.5 x 10^{-6} (Singh and Klingmüller, 1986), and for various strains of *Rhizobium meliloti* it ranged between 4 x 10^{-3} and 4 x 10^{-4} (Sevaraj and Iyer, 1983). Thus the transconjugation frequency was lower for WPW-S28 than for these other microorganisms, particularly the *R. meliloti*. Owen and Ward (1985) observed a transconjugation frequency of 10^{-7} for Tn5 in *Azotobacter beijerinckii* using a suicide vector other than pGS9. These results were similar to the transconjugation frequency observed for WPW-S28.

After cell mating mixtures were plated on GK agar, approximately 2 wk was required for colonies to develop. This was not particularly surprising; WPW-S28 generally required at least 1 wk of incubation before colonies appeared on rich agar plates such as LB agar, and longer on plates with a less complete nutrient content. Colonies were picked from the GK agar plates and transferred to fresh GK agar, using a grid of 50 colonies per plate. These colonies were allowed to develop, then were replica plated onto PAT medium containing 50 mg/L kan, and on GK agar. Approximately 75% of the original picked colonies grew well enough to be transferred by replica plating. The GK agar was used to ensure that these colonies were not *E. coli*, which could not grow on this medium. PAT medium was used as a screening device; colonies in which the sulfolane-degrading genes had been disrupted should appear negative for sulfolane degradation on PAT medium, which was designed to detect an end product of aerobic sulfolane mineralization, acid (Section 3.4.1). WPW-S28 colonies which had Tn5 integrated into genes not involved in sulfolane degradation were expected to produce positive colonies on PAT medium. The presence of kan prevented WPW-S28 which did not have Tn5 from growing.

A total of 250 replica plates, each initially inoculated with 50 WPW-S28 kan^R colonies, were screened. Approximately 75% of the colonies grew on the original GK patch plates, resulting in a total of 9375 kan^R colonies being tested for their ability to utilize sulfolane. Approximately 20 colonies appeared to be negative for sulfolane degradation; these were transferred to fresh GK agar. Once these colonies had grown, they were replica plated onto GK agar, PAT medium containing 50 mg/L kan, and GK agar supplemented with 30 mg/L chloramphenicol (cm), to ensure that Tn5 had integrated into the chromosome. The isolate WPW-S28 was not identified, therefore it was impossible to predict whether this organism was likely to be able to support pGS9 as a free-living plasmid, although due to the narrow host specificity of the plasmid this was not likely to have occurred. No colonies grew on medium supplied with cm, however most of the colonies were able to utilize sulfolane, as demonstrated by the development of yellow halos on PAT medium. PAT medium was not selective for sulfolane-degrading organisms. Without selective pressure, the reversion rate for WPW-S28 no longer able to degrade sulfolane to a sulfolane-degrading phenotype was surprising. Careful observation of the selected colonies over time demonstrated that the ability to degrade sulfolane was sometimes present and sometimes absent; streaking a colony that appeared to be unable to degrade sulfolane on PAT agar would produce some colonies that could use the compound and others that could not. Further observations of WPW-S28 that had not been mutagenized and did not demonstrate kan^R demonstrated that a colony unable to degrade sulfolane would occasionally appear. These colonies were not contaminants; they displayed the same microscopic characteristics as WPW-S28 and appeared the same when streaked on rich medium; also, they could be streaked on PAT medium and would give rise to some colonies that were again able to degrade sulfolane. Due to the complications experienced with attempts to mutagenize WPW-S28, this project was not continued. Transposon mutagenesis might be useful with a different pure, sulfolane-degrading culture that demonstrated fewer complex growth characteristics.

Later experiments to identify the isolate WPW-S28 suggested that it was *V. paradoxus*, or an undescribed member of this genus. Di Giovanni et al. (1996) demonstrated that 32 environmental isolates identified as *V. paradoxus* all showed frequent gene rearrangements, resulting in variations in biodegradative activities by these isolates. Given this information, it was not unreasonable to observe the variations seen in WPW-S28 mutagenized with Tn5 during this experiment. The *V. paradoxus* isolates described by Di Giovanni et al. (1996) were also demonstrated to contain a number of plasmids, some large, which may have encoded catabolic genes for 2,4-D degradation. Although WPW-S28 was tested for the presence of plasmids, a relatively vigorous alkaline lysis method was employed; this method may not have detected large plasmids. A future experiment for isolating sulfolane-degrading genes from WPW-S28 could be attempts to detect large plasmids by gentle plasmid isolation techniques.

D.2 Existence of extractable metabolites in culture supernatants

The compounds produced as a result of sulfolane degradation were predicted to be small and highly polar (Section 3.8.2). They were not detectable using the sulfolane or acetate detection GC methods. The aqueous injection GC method for sulfolane detection was useful for the detection of compounds such as sulfolane, sulfolene, phenol and butane sulfonic acid (data not shown). The products of sulfolane biodegradation were not detectable by this GC method, therefore were likely to have had different properties than the compounds listed. Analysis of culture supernatants using the acetate analysis GC method produced some visible peaks, however these were small and were not reproducible between different cultures of the same organism (data not shown). All peaks detected by this method were between ones observed for formic and butyric acids, therefore they likely contained 1-4 C, however they could not be identified using this GC method. Methods used for this work are described in Section 2.13.2.

Extraction of cell supernatants with ethyl acetate during or after sulfolane degradation resulted in the recovery of sulfolane, but not of any other compounds detectable with an FID or FPD when extracts were dried under N_2 and analyzed using the DB-5 capillary column GC method (data not shown). Due to the difficulty in detecting metabolites in culture extracts, it was felt that derivatization of these metabolites would increase the likelihood that they could be detected. The existence of such metabolites was demonstrated by the presence of dissolved organic C in culture supernatants after sulfolane removal was complete, and no further CO_2 production for these active cultures observed during prolonged incubation (Section 3.8.2).

D.2.1 Model compound detection

Three derivatization methods were used: methylation with diazomethane or with methyl iodide (iodomethane), or trimethylsilylation with N,O-bis(trimethylsilylacetamide) (BSA). Four model compounds were tested; these all contained more C than sulfolane; thus their detection was more likely and the analytical methods could be more reliably compared, using various derivatization methods. All the samples were analyzed using a GC equipped with a DB-5 capillary column, a FID and a FPD. The temperature program is described in Section 2.2.1.1. Diazomethane-derivatized 3-hydroxysulfolane produced a large, poorly defined S-containing peak at approximately 14.5 to 16 min, as well as two substantially smaller S-containing peaks at 8 min and 11 min (data not shown). It is likely that the large peak represented completely methylated 3-hydroxysulfolane, and the two smaller peaks, which appeared earlier in the time program, were representative of partially derivatized substrate. Sulfolane, when derivatized with diazomethane, produced a similar large peak at approximately 16-17 min, while untreated sulfolane appeared at 8-9 min, using this temperature program. Thus, the peak appearing at 8 min in the 3-hydroxysulfolane sample may have been sulfolane. However, this peak was not identified.

Benzenesulfonic acid, 1-decanesulfonic acid, and 2-sulfobenzioc acid could not be detected by GC after derivatization with iodomethane. After derivatization with diazomethane, 1-decanesulfonic acid was detected in the form of one large peak at 24 min and a much smaller peak at 25 min. Benzenesulfonic acid was not detected after derivatization with diazomethane.

Benzenesulfonic acid could not be detected after methylation (if methylation of the compound had occurred). Both 3-hydroxysulfolane and 1-decanesulfonic acid were detectable after derivatization with diazomethane, although 1-decanesulfonic acid was not detected after iodomethane derivatization. These results suggested that it might be possible to detect smaller straight chain sulfonic acids or oxidized products of sulfolane by derivatizing culture supernatants with diazomethane. Iodomethane derivatization involved dissolving samples in benzene, then heating them overnight in the presence of iodomethane, in a reflux apparatus, therefore diazomethane derivatization method was simpler, as well as more effective in the detection of the model compounds.

D.2.2 Derivatization of acidified WPW-S28 supernatants with diazomethane

A series of cultures of WPW-S28 were incubated for the C and S balance for sulfolane degradation by this culture (Section 3.8.2). There were a total of 15 cultures incubated for CO_2 analysis; triplicate samples were acidified to pH < 2 after 0, 24, 48, 72 and 264 h of incubation. These cultures were centrifuged to remove cells and cell debris, filter sterilized with a 0.22 μ filter and the supernatants for each time point were combined and freeze-dried. The soluble organic C resulting from sulfolane degradation that was present in the combined supernatants for each time point was calculated to be 5.16 mg, 4.02 mg, 1.56 mg, 1.80 mg and 1.56 mg, from time zero to the end of the incubation, respectively, based on the measurements taken previously (Figure 3.22). The freeze-dried sample for each time point was derivatized with diazomethane in diethyl ether, dried under N_2, dissolved in dichloromethane and analyzed using a GC equipped with a FID and a FPD. A S-containing peak other than sulfolane was observed in the 24-h sample, but it eluted too close to the sulfolane-containing peak to be analyzed further. The sulfolane peak was observed at 8 min, and the unknown peak at 10 min, on the shoulder of the sulfolane peak (data not shown). This peak was not observed in any of the washed cell experiments carried out with WPW-S28 (Section D.2.5), therefore it may have been an artifact of the experimental process or a contaminant compound introduced during the preparation or derivatization of the sample.

D.2.3 Proton NMR of sulfolane and water incubated with washed WPW-S28

Combourieu et al. (1998) developed a ^{1}H-NMR method for detecting the intermediate compounds in the morpholine degradation pathway of *Mycobacterium aurum* strain MO1. Morpholine is a highly water-soluble 6-membered N and O heterocyclic compound, which is employed in several industrial processes. The N and O atoms are at opposite sides of the 6-membered ring; two C atoms lie between each hetero-atom. The hypothetical pathway for its biodegradation was cleavage of the ring next to the N atom, followed by cleavage next to the O atom, which resulted in the production of ethanolamine and glycolate (Combourieu et al., 1998). Cells were grown on morpholine, harvested, then incubated with morpholine in a low salt buffer (Combourieu et al., 1998). The supernatant was filter sterilized, supplemented with D_2O (10% v/v) and subjected to ^{1}H-NMR. Using this method, the authors were able to detect glycolate and 2-(2-aminoethoxy)acetate during morpholine biodegradation. The C and S balance time course for WPW-S28 biodegradation of sulfolane suggested that some soluble organic C compounds were produced during sulfolane removal from the culture supernatant, and that some of these compounds were converted to CO_2 over time (Section 3.8.2). It was possible that the products of sulfolane biodegradation by the pure culture WPW-S28 could be detected using a similar protocol to that used by Comborurieu et al. (1998).

Proton NMR analysis of samples required that the culture supernatant contain little or no salt, therefore WPW-S28 cultures harvested during the logarithmic phase of growth were washed in water, then used as an inoculum in water containing sulfolane. Although some cell lysis due to this treatment was predicted, some part of the population was expected to survive the osmotic shock. Cells were centrifuged, then the supernatant was filter sterilized to remove all cell debris before NMR analysis. Samples were freeze dried, dissolved in D_2O, and analyzed by A. Otter (Dept. of Chemistry, University of

Alberta, Edmonton, AB). Sulfolane was easily detected by this method (Figure D2). Some mixed products other than sulfolane were detected after sulfolane degradation had commenced (sulfolane degradation was monitored by GC analysis), however these compounds were not identifiable because there was more than one product present, and they were mixed. The only conclusion that could be made from the NMR analysis was that no aldehydes were produced during sulfolane degradation by WPW-S28 (A. Otter, personal communication).

D.2.4 Solid phase extraction and GC-AED analysis of WPW-S28 washed cell supernatants

Aqueous samples from a WPW-S28 washed cell time course experiment (Section C.2.5) were analyzed by J. Hoyle (Dept. of Chemistry, University of Alberta, Edmonton, AB), using a solid phase extraction technique followed by GC analysis with an atomic emission detector (AED). Samples were extracted using a fiber-coated syringe system. Compounds present in the aqueous sample would bind to the fiber over a period of 5 to 10 min, after which the solid phase was inserted directly into the GC injection port. The compounds adhering to the fiber were released by the heat of the injection port, and could enter the column. Sulfolane was detected by this method (Figure D3), however no other compounds were readily detected (data not shown). It is likely that the products of sulfolane metabolism by WPW-S28 were too small and/or too polar to sorb to the fiber used in this extraction method, or that they were present in such small quantities that they could not be detected. Another possibility is that the sorbed products were not thermally desorbed from the fiber on the syringe in the injection port, or that these products were not volatile enough to move through the GC column.

Figure D3 shows a 50 mg/L sulfolane standard. To prepare the injection syringe, 1 mL sulfolane standard was used, representing 0.05 mg sulfolane. Samples from the washed cell experiments used for the solid phase extraction contained 0.12 mg sulfolane at time zero. A total of less than half this amount, 0.052 mg sulfolane, was used by WPW-S28 during the experiments. In previous experiments, WPW-S28 converted 62% of sulfolane to soluble organic C (Section 3.8.2), therefore a maximum of 0.032 mg of products remaining from sulfolane might have been present in the sample used for solid phase GC analysis. Because $SO_4^=$ was released relatively quickly after sulfolane removal by WPW-S28 (Section 3.8.2), these compounds may not have contained an S atom. Since approximately 0.032 mg sulfolane products were predicted to be in this sample, only 0.013 mg C may have been involved in soluble compounds present in the

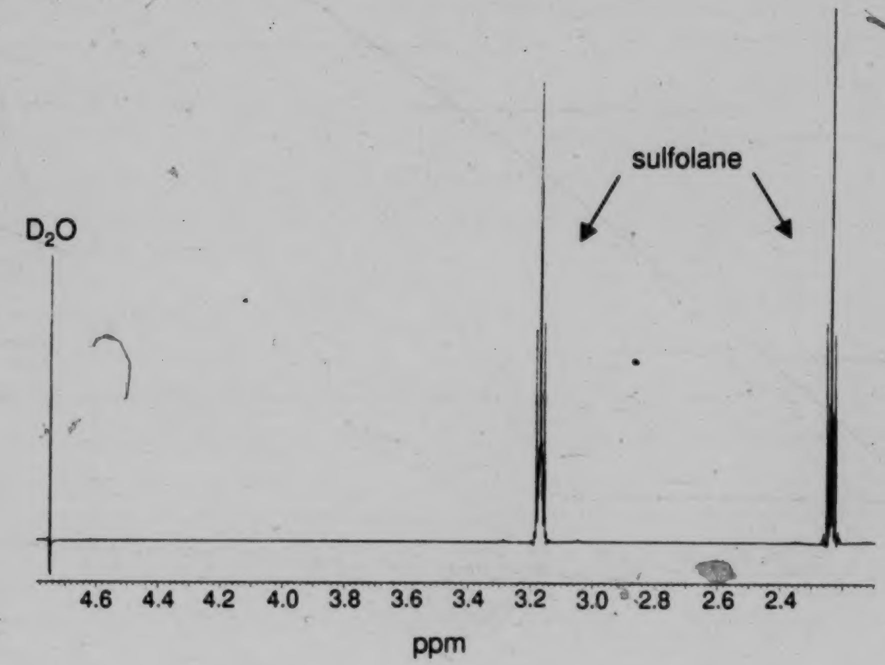


Figure D2. Sulfolane detection by ^{1}H-NMR.

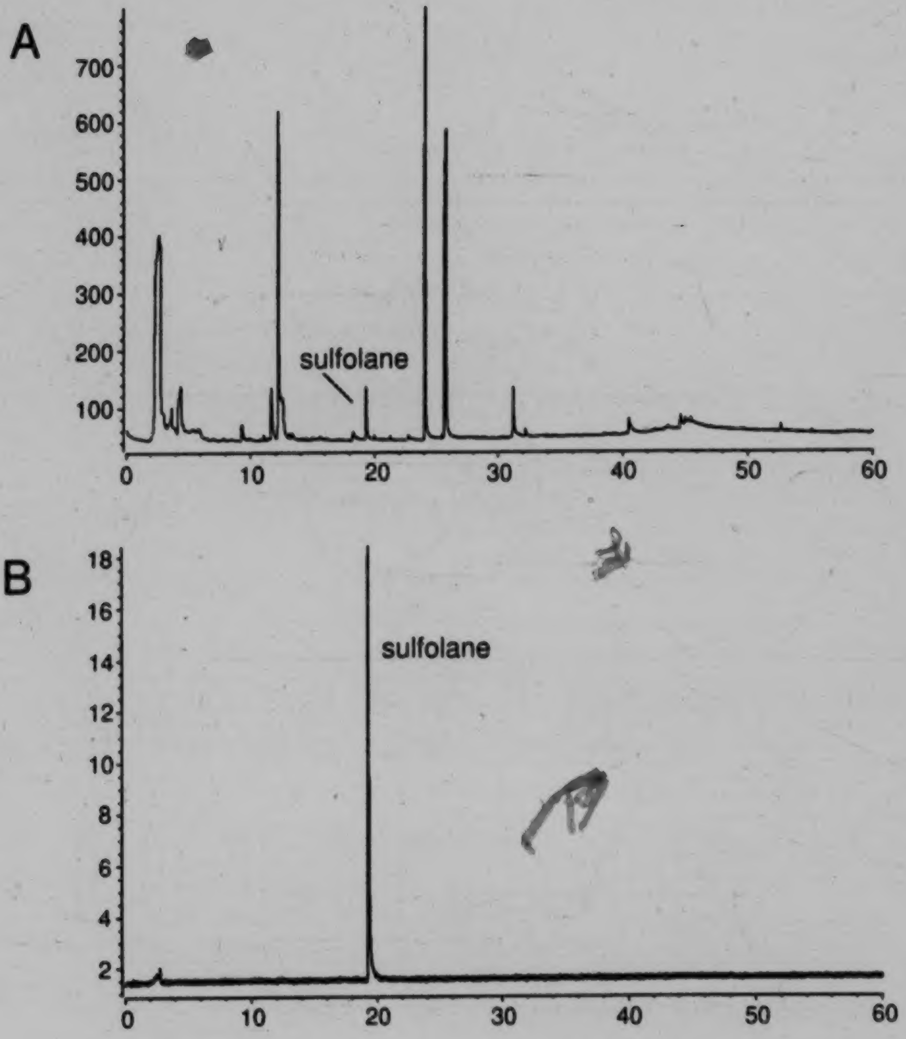


Figure D3. Sulfolane detection by GC-AED analysis of a 50 mg/L standard obtained using solid-phase extraction techniques. A. C trace (60 min). B. S trace (60 min).

sample. The NMR results (Section D.2.3) suggested that sulfolane degradation by WPW-S28 resulted in more than one product compound. Even if such compounds were trapped by the solid phase extraction system, their quantities may have been too small to detect.

D.2.5. Derivatization of WPW-S28 washed cell extracts with BSA

A series of washed cell experiments in which cultures of WPW-S28 that had been harvested during the logarithmic phase of growth on sulfolane and resuspended in phosphate buffer (50 mM KH_2PO_4 in deionized, distilled water) were done. In each case, cultures were harvested at various time points after the addition of sulfolane, centrifuged, and the supernatant filter sterilized. Duplicate cultures were used to provide supernatants that were brought to pH < 2 or pH > 11. The pH of each filter-sterilized culture supernatant was adjusted, then samples were freeze-dried and derivatized with BSA. BSA was selected because it would result in a much larger product (trimethylsilylation of each reactive group on compounds in the sample rather than methylation) than the other derivatization agents used (diazomethane and iodomethane). Samples were analyzed by GC.

In total, five series of experiments were done. No compounds, including sulfolane, were detected under basic conditions. Under acidic conditions, sulfolane was easily detected by both the FID and the FPD. Some C-containing compounds were occasionally detected in samples where sulfolane degradation had occurred, however the detection of these compounds was not reproducible between experiments, nor were they detected at more than one time point in each experiment. S-containing compounds other than sulfolane were not detected in any of the samples.

These results suggest that either BSA was not able to interact with the sulfolane degradation products in these samples, or that even when these compounds were derivatized they were at concentrations too low to detect. The possibility that more than one degradation product may exist, which was suggested by the NMR analysis of WPW-S28 culture supernatant, would support the hypothesis that any existing sulfolane metabolite was present at very low concentrations. For example, 41 mg/L sulfolane was removed from one washed cell culture in which the maximum sulfolane removal for that washed cell experiment had occurred. In the 25-mL culture, this amount of sulfolane represented 16.4 mg C. Given the results of the C and S mass balance for WPW-S28 grown on sulfolane (Section 3.8.2), only 26% of this C, or 4.3 mg, may have been present as soluble organic C, which may have been too low a concentration to detect. Larger scale experiments would be required to ameliorate this problem.

A second possibility was that the compounds produced by sulfolane biodegradation were volatile, but were not CO_2 and were not detectable by GC-TCD analysis of headspace gas in the serum bottle cultures. Biodegradation of sulfolane by WPW-S28 always resulted in a strong odor reminiscent of a sulfurous compound, suggesting that a volatile product containing S may have been formed. It is unlikely that a volatile, S-containing product, could account for the unidentified C products of sulfolane degradation, since a great deal of the unidentified C products were present as soluble C in the culture supernatant. However, the untraceable S (Section 3.8.2) and a small portion of C may be released in the form of a volatile compound. Further research is required to determine whether such volatile compounds are produced by WPW-S28, and to identify the soluble C metabolites of sulfolane biodegradation by this culture.